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Release and cellular uptake of anticancer drugs encapsulated in apoferritin

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.” (*Arthur Schopenhauer*)

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Yesterday I was starting, today I am delivering. The time was short, the list of those who directly or indirectly contributed to the completion of this work is not. I want to use this opportunity to thank the all people without whom I would not be able to develop this work.

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Resumo

O cancro continua a ser uma doença letal. Inicialmente silencioso, vai crescendo e expandindo a sua área, podendo até invadir e começar um novo foco noutros tecidos. Os métodos atuais de tratamento estão dependentes do tipo e características do tumor em questão. Porém, de uma forma geral, podem ser divididos em cirurgia, radioterapia e quimioterapia. Cada um destes métodos é eficaz em determinadas circunstâncias, porém tem também as suas desvantagens, como falta de eliminação total do tumor, variados efeitos colaterais e potencial agressivo para o paciente.

A quimioterapia é geralmente o tratamento mais eficiente. Diminui a quantidade de células cancerígenas, a possibilidade de reincidência e a taxa de morte da doença. Assim sendo, é utilizada como terapia individual com sucesso, mas também pode preceder uma cirurgia, que ocorrerá após diminuição da massa celular. Apesar do sucesso, existe um número de fármacos que pelas suas características, apesar de altamente eficazes para a eliminação do tumor, não podem ser dados ao paciente. É fundamental obter um sistema de transporte, que proceda à proteção do fármaco e o consiga entregar ao tumor, ou mesmo auxiliar na sua internalização pelas células tumorais.

A ferritina é biocompatível, estável e capaz de internalizar vários compostos. Além disto, como as células cancerígenas superexpressão o recetor da ferritina (TfR1), esta emerge como um potencial veículo de transporte de fármacos direcionados para células tumorais.

Motivado pela enorme procura de novas terapias e estratégias contra o cancro e pelas maravilhosas propriedades estruturais da ferritina, este trabalho tem como objetivo comprovar a encapsulação de fármacos e o potencial como sistema de entrega de fármacos, a células cancerígenas, da ferritina. Assim, a cisplatina foi dissolvida em água, promovendo carga positiva no composto e aumentando a eficiência de encapsulação. O imatinib foi encapsulado com recurso à capacidade de a ferritina dissociar-se e reassociar-se. O efeito metabólico verificado pelo ensaio da resazurina nas linhas celulares K562 e Panc1, que correspondem a linhas de leucemia mieloide crónica e adenocarcinoma pancreático, respetivamente.

Os resultados obtidos são promissores. Análises TEM provaram a reassociação e as conjugações FI e FC diminuíram o valor de IC_{50} para 0.125 µM e 6.25µM, respetivamente. Tal demostra o elevado potencial da ferritina para transporte de fármacos. Serão necessários trabalhos adicionais de modo a continuar estes estudos, utilizando outras linhas celulares e modelos animais, com o objetivo de confirmar a atividade anticancerígena aumentada e verificar o mecanismo de ação destas conjugações.

Palavras-chave: Leucemia Mieloide Crónica, Adenocarcinoma Pancreático, Ferritina, Transporte de Fármacos, Imatinib, Cisplatina

Abstract

Cancer is a lethal disease. Silent in the beginning, cancer will grow and expand its area, possibly invading and spreading into other tissues. Current treatment methods are dependent on the tumour type and characteristics. However, they may be divided into surgery, radiotherapy and chemotherapy. Each method is effective on certain circumstances, however they also have their disadvantages, like lack of total tumour removal, diverse side effects and aggressive potential for patient.

Chemotherapy is generally the most efficient treatment. Chemotherapy decreases the cancer cells number, the recurrence possibility and the disease death rate. Therefore, it is used as a successful therapy by itself, however it may also precede a surgery, which will occur after a cell mass decrease. Despite the success, there are several drugs that by their characteristics, although highly effective for tumour elimination, can not be delivered to the patient. It is fundamental to obtain a transport system that protects the drug and is able to deliver the drug to the tumour, or even aid in its internalization by the tumour cells.

Ferritin is biocompatible, stable and is able to internalize several compounds. Furthermore, as tumour cells tend to overexpress its receptor (TfR1), ferritin emerges as a potential tumour cells targeted drug delivery vehicle.

Motivated by the huge cancer new therapies and strategies demand and by the marvellous structural properties of ferritin protein, this work aims to ascertain ferritin drug encapsulation and cancer cells delivery system potential. Thus, imatinib and cisplatin were internalized into ferritin. Cisplatin was dissolved in water, to promote a positive charge gain and improve the encapsulation efficiency. Imatinib was incorporated through the exploiting of the dissociation-association ferritin cage capacity. The metabolic effect was accessed by resazurin assay on the K562 and Panc1 cell lines, which correspond to chronic myeloid leukaemia and pancreatic adenocarcinoma cellular lines respectively.

Promising results were obtained. TEM analysis proved the ferritin cage reassembly and the FI and FC conjugations decreased the IC_{50} to 0.125 μM and 6.25 μM, respectively. This demonstrates the high potential of ferritin drug delivery systems. Further work will need to be carried out in order to continue these studies, using other cell lines and animal models, aiming to confirm the enhanced anticancer activity and verify the mechanism of action of these conjugations.

Keywords: Chronic Myeloid Leukaemia, Adenocarcinoma Pancreatic Cancer, Ferritin, Drug Delivery, Imatinib, Cisplatin

Figures

Tables

Abbreviations

AAFP - American Academy of Family Physicians

ABL - Abelson cytoplasmic tyrosine kinase

ALL - Acute lymphoblastic leukemia

AML - Acute myeloid leukemia

Bax - Bcl-2-associated X protein

Bcl-2 - B-cell lymphoma 2

Bcl-x_L- B-cell lymphoma extra large

BCR - Breakpoint cluster region

BRCA2 - Breast cancer 2

CAPS - International Cancer of the Pancreas Screening Consortium

CBC - Complete blood count

CD - Circular dichroism

CML - Chronic myeloid leukaemia

CPC - Cardiovascular progenitor cells

CT - Computed tomography

CYP - Cytochrome P450

DDI - Drug-to-drug interaction

DNA - Deoxyribonucleic Acid

ELN - European LeukemiaNet

ESMO - European Society of Medical Oncology

FAS - First apoptosis receptor

FC1 - Ferritin-cisplatin conjugate in 0,9% NaCl

FC2 - Ferritin-cisplatin conjugate previously in 0,9% NaCl then dissolved in water

FC3 - Ferritin-cisplatin conjugate in water

FCT1 - Cisplatin-NMOF not urea treated

FCT2 - Cisplatin-NMOF urea treated

FI - Ferritin-imatinib conjugation

GLUT-1 - Glucose transporter 1

GVHD - graft-versus-host-disease

GRAS - Generally regarded as safe

HLA - Human leukocyte antigen

HSCT - Hematopoietic stem cell transplantation

IC_{50} - Half maximal inhibitory concentration

Igf1/2 – Insulin-like growth factors 1/2

1L-2 - Interleukin 2

Kras - Kirsten rat sarcoma viral oncogene homolog

MAP - Mitogen activated protein

MHC - Major histocompatibility complex

NCCN - National Comprehensive Cancer Network

NMOF - Nanoscale metal–organic frameworks

NOXA - Phorbol-12-myristate-13-acetate-induced protein 1

PanIN - pancreatic intraepithelial neoplasia

PDGFR - Platelet-derived growth factor receptor

Ph+ - Philadelphia positive chromosome

PUMA - P53 upregulated modulator of apoptosis

QPCR - Quantitative reverse transcription polymerase chain reaction

RES - Reticuloendothelial system

RPMI - Roswell park memorial institute medium

siRNA – Small interference ribonucleic acid

SPARC - Secreted protein acidic and rich in cysteine

STAT - Signal transducers and activators of transcription

TEM - Transmission electron microscopy

TGFβ - Transforming growth factor beta

TSP-1 - Thrombospondin-1

TKI - Tyrosine kinases inhibitors

USPSTF - U.S. Preventive Services Task Force

VEGF - Vascular endothelial growth factor

WBC – White blood cells

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1. Literature Review

1.1. Cancer

1.1.1. Introduction

Organism development, cell growth and differentiation and even maintenance of vital functions involve exact regulation of the time and location of cell division and programmed cell death, or apoptosis.

The broad term cancer is given to a group of diseases characterized by an abnormal growth and formation of cells populations, that can start almost anywhere in the human body, in a multistage process which may last many years (Paduch, 2015). In fact, since 1958 tumorigenesis was already thought as a multistep process (Fearon & Vogelstein, 1990). The origin of this group of diseases is not fully understood and neither its way of development. Many theories have been developed through the years attempting to explain the cancer causes, however no consensus exists. Cancer is mostly caused by mutation (permanent alteration in the nucleotide sequence) or, by some other abnormal activation of cellular genes, that control cell growth and/or mitosis. Thus, lead to cell cycle deregulation and out of control proliferation (Glickman & Radman, 1980; Helleday, 2013). According to one of the most important models of carcinogenesis, the somatic mutation theory, these DNA changes, are mutations in oncogenes and tumour-suppressor genes. Oncogenes mutations would lead to cell proliferation while tumour-suppressor genes mutations would lead to the inhibition of the cell death or cell-cycle arrest (Pup et al., 2016).

Regardless the affected genes, mutations occur naturally in our cells, even though DNA chromosomal strands are replicated, by DNA polymerases with great precision. Furthermore, the proofreading process cuts and repairs any abnormal DNA strand, even before the mitotic process may proceed (Martinocorena & Campbell, 2015). However, as numerous new cells are formed each year, there is a probability of a significant mutation to appear on a newly formed cell, each every few million, at some point on mitosis. This probability can be further increased by the exposition to certain chemical, physical or biological factors like ionizing radiation (X-rays, γ-rays), carcinogens (cigarette smoke compounds, endocrine disruptors), virus or hereditary factors (Clapp, Jacobs, & Loechler, 2009).

Although mutations may occur, fortunately not all ever lead to cancer. Only a minute fraction of the mutated cells in the body ever reaches the tumorigenic stage. Some of the mutants have, in fact, less survival capacity than normal cells and simply die, while others do not present phenotypic alterations (Douglas Hanahan & Weinberg, 2011). Most mutated cells still have normal feedback controls that prevent excessive growth. These cells just appear in the normal tissues without further complications (A. Y. Chow, 2010). Furthermore, while a specific oncogene might promote rapid non-stop cell divisions other, for example, would be needed to form new blood vessels. Therefore, to cause cancer is

usually required the mutated activity of different genes simultaneously (A. Y. Chow, 2010). However, as these mutations may form abnormal proteins within the cell bodies, due to the altered genes, the immune system may recognize these mutants and be activated. Antibodies or sensitized lymphocytes that react against cancer, destroying the cancerous cells, are then formed (Marcus et al., 2014). In fact, in people whose immune systems have been suppressed, such as in those taking immunosuppressant drugs after kidney or heart transplantation, the probability of a cancer's developing is multiplied as much as fivefold (Khonsary, 2017) , and there is a striking increase of certain cancers in immunocompromised individuals (Schulz, 2009).

Most tumours can be clinically classified as benign or malign. The cells of the malignant tumours are pleomorphic, varying in size and shape. Furthermore, these cells are less differentiated, or anaplastic than their benign counterparts (Paoloni & Khanna, 2007).

1.1.2. Cancer Hallmarks

A series of traits must be acquired in order to cells become malignant. These are distinctive and complementary capabilities that enables the growth of the tumour and its metastasizing ability, providing a solid basis for cancer understanding. Replicative immortality, proliferative signals sustain, cell death resistance and evasion to growth suppressors are the main traits and, must be achieved, figure 1 (Douglas Hanahan & Weinberg, 2011). Cancer cells may also gain the capability of inducing angiogenesis and invading other tissues (metastaticity capacity).

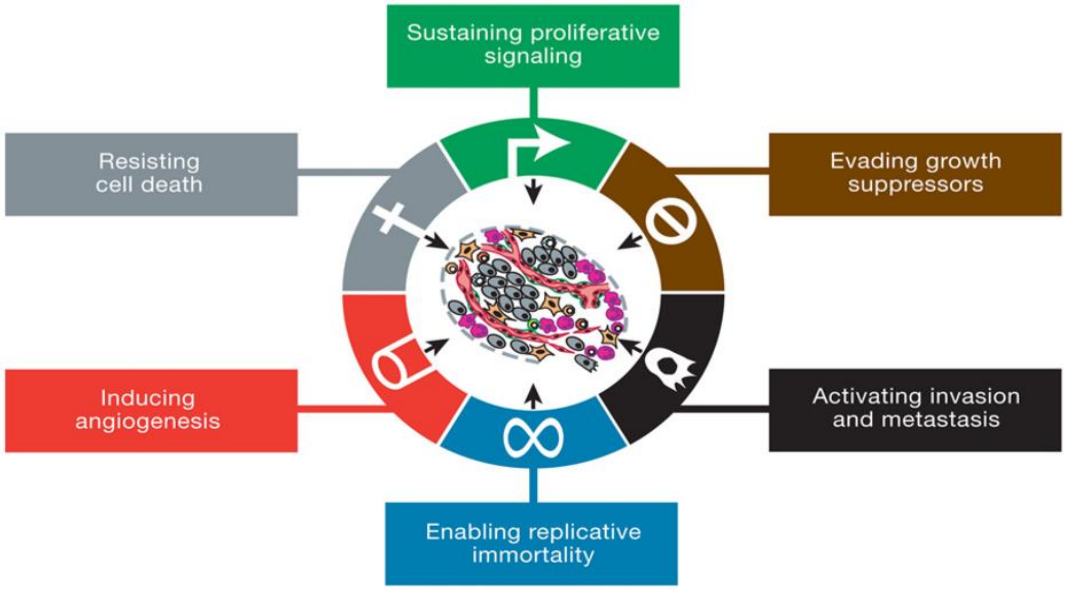


Figure 1 Cancer hallmarks organized in six categories (Douglas Hanahan & Weinberg, 2011)

a) Proliferative Signalling

Normal tissues control the production and release of growth-promoting signals. Thus, instructing the tissue progression through cell growth and division cycle control. This way, homeostasis and cell number, as well tissue structure and function maintenance, are ensured. Tumours, however, generate many of their own growth signals, reducing their dependence of the normal tissue microenvironment, becoming masters of their own fate (D Hanahan & Weinberg, 2000).

Cancer cells can acquire the capability to maintain proliferative signalling in several different ways, such as the production of growth factors that bind cell-surface receptors, typically containing intracellular tyrosine kinase domains (signals are then emitted via branched intracellular signals pathways). Another way is the release of signals to stimulate normal cells, within the supporting tumour associated stroma, which respond by supplying the cancer cells with the required growth factors (Douglas Hanahan & Weinberg, 2011). Furthermore, cancer cells control the expression of extracellular matrix receptors (integrins), favouring ones that transmit pro-growth signals.

The transduction of the external signals influences the cell behaviour, enhancing motility, apoptosis resistance and entrance into the cell division cycle.

b) Evading growth suppressors

To maintain cellular quiescence and tissue homeostasis, multiple anti-proliferative signals are required. Cells must also circumvent powerful programs that negatively regulate cell proliferation. Both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the nearby cells surfaces, are anti-proliferative signals (D Hanahan & Weinberg, 2000).

The antigrowth signals can block proliferation by two distinct mechanisms:

- Cells may be forced out of proliferative cycle into quiescence state (G_0) from which they may remerge when extracellular signals allow;
- Cells may be induced to permanently give up their proliferative potential by being forced into post-mitotic states, typically associated with acquisition of differentiated-associated traits.

To prosper, cancer cells must evade these anti-proliferative signals. At the molecular level, many of the anti-proliferative signals are funnelled through the hypophosphorylated retinoblastoma protein and its two relatives, the retinoblastoma-like protein 1 and 2 (D Hanahan & Weinberg, 2000).

Functional retinoblastoma protein may be lost through mutations of the gene or through the soluble signalling molecule TGFβ (transforming growth factor beta), which stops the phosphorylation, thus inactivating the retinoblastoma protein.

c) Resisting cell death

The tumour cell population capability to increase in numbers is determined not only by the cell proliferation rate but also by the cell attrition rate. Apoptosis represents the major source of attrition (D Hanahan & Weinberg, 2000). Apoptosis, triggered by physiological stresses that cells experience during tumorigenesis and anticancer therapy, was established as a natural barrier to cancer development in several functional studies (Adams & Cory, 2007).

The apoptotic machinery is composed of both upstream and downstream effectors components (Adams & Cory, 2007). The regulators are divided into two major circuits, one receiving and processing extracellular death-inducing signals, the extrinsic apoptotic program, involving the FAS (Type-II transmembrane protein) ligand/FAS receptor, and other sensing and integrating a variety of signals of intracellular origin, the intrinsic program.

These programs culminate in the caspase 9 and 8 activation, which proceeds to initiate a cascade of proteolysis involving effectors caspases that are responsible for running apoptosis. Cell is progressively disassembled and then consumed, both by its neighbours and phagocyte cells. The intrinsic apoptotic program is more widely implicated as a cancer pathogenesis barrier (D Hanahan & Weinberg, 2000; Douglas Hanahan & Weinberg, 2011).

Many of the signals that elicit apoptosis converge on the mitochondria, which in turn respond to pro-apoptotic signals by releasing cytochrome C, a potent apoptosis catalyst that will induce multiple cellular changes apoptosis associated (D Hanahan & Weinberg, 2000).

Several abnormality in sensors, that play key roles in tumour development, have been identified (Adams & Cory, 2007; Lowe, Cepero, & Evan, 2004). Most prominent is in a DNA-damage sensor that functions via the P53 tumour suppressor gene, inducing apoptosis by NOXA (Phorbol-12-myristate-13-acetate-induced protein 1) and PUMA (P53 upregulated modulator of apoptosis) proteins expression up-regulating, in response to substantial DNA breaks and chromosomal abnormalities (D Hanahan & Weinberg, 2000).

The loss of a pro-apoptotic regulator through P53 tumour suppressor gene mutation is seen in more than 50% of the human cancers (Harris, 1996a), and results in P53 protein inactivation, which leads to the deletion of a key component of the DNA damage sensor. Therefore, inducing the apoptotic effectors cascade (Harris, 1996b). Signal evoked by other abnormalities, including hypoxia and oncogene hyper-expression, are impaired at eliciting apoptosis when P53 function is lost. Additionally, apoptosis inhibitors may also have an increased expression (Bcl-2, Bcl-x_L) or a proapoptotic factors down regulation (Bax, Bim, Puma). It is also possible to have an increased expression of survival signals (Igf1/2) or short-circuiting the extrinsic ligand-induced death pathway (D Hanahan & Weinberg, 2000).

Tumour cells evolve a variety of strategies to limit or circumvent apoptosis and the multiplicity of these evading mechanisms presumably reflects the diversity of apoptosis inducing signals that cancer cells populations encounter during their transformation to the malignant state.

d) Enabling replicative immortality

It is widely accepted that cancer cells require infinite replicative potential in order to become tumours. This capability marks a clear contrast comparatively with "normal cells", which are only able to pass through a limited number of cell growth and divisions cycles. This limitation has been associated with two different barriers to proliferation: senescence and crisis.

Senescence is an irreversible entrance into a non-proliferative but viable state, and crisis involves cell death. Accordingly, when cells are propagated in culture, repeated cell division cycles lead to senescence after what cells that succeed in circumventing this barrier, progress to crisis phase in which the great majority of cells die. On rare situations, cell may emerge from a population in crisis and exhibit unlimited replicative potential. This transition has been called immortalization, an attribute that most established cell lines possess since they can proliferate in culture without evidence of either senescence or crisis (D Hanahan & Weinberg, 2000; Douglas Hanahan & Weinberg, 2011).

Telomeres protect the end of chromosomes, and its length underlies the number of times a cell can have successive division. At each division a decrease in its length is observed. Thus, at a certain moment, telomeres will lose their function of protection causing cell death.

Repetitive DNA sequences may be added at the end of telomeres by telomerases. Without this nucleic material repetition addition, a cell can only divide 50 to 60 times, after what it becomes senescent. However, with telomerase any cell can divide without limit, like is observed in tumour and immortalized cells.

This telomerase activity, both in spontaneously immortalized cells or in cell engineered to that effect, is correlated with an induction to senescence and crisis resistance. Conversely, telomerase suppression leads to telomers shortening and the activation of the proliferative barriers (D Hanahan & Weinberg, 2000; Douglas Hanahan & Weinberg, 2011).

e) Inducing angiogenesis

Virtually all cells in a tissue should reside within 100 μm of a capillary blood vessel, safeguarding the proper oxygen and nutrients supply, essential for cell function and survival. This is ensured during organogenesis, by coordinated growth of vessels and parenchyma and carefully regulated once the tissue is formed. Without angiogenic capability, even with aberrant proliferative lesions, the expansion is restricted (Douglas Hanahan & Folkman, 1996).

The balance between positive and negative signals will determine the fate of the formation of new vessels, promoting or blocking angiogenesis. The angiogenic initiation signals are related to the vascular endothelial growth factor (VEGF) and acidic/basic fibroblast growth factors which bind to transmembrane tyrosine kinase receptors in the endothelial cells. Thrombospondin-1 (TSP-1), on other hand, is an angiogenic inhibitor which binds to cluster of differentiation 36, a transmembrane receptor on endothelial cells (Veikkola et al., 2000).

The growing tissue that is the tumour will require vast amounts of oxygen, nutrients and metabolic waste elimination provided by vasculature. Oppositely to the angiogenic quiescence normal adulthood state, in tumours angiogenesis (formation process of new blood vessels from pre-existing ones) is turned on transiently helping to maintain the expanding growth (D Hanahan & Weinberg, 2000).

Tumours may produce their own angiogenic factors or influence the surrounding normal cells to the synthesis and secretion of such factors. Despite being formed, the new blood vessels are typically aberrant and more permeable. In fact, the tumour neovascular system is marked by precocious capillary development, convoluted and excessive vessel branching, deformed and enlarged vessels, erratic blood flow, leakiness and abnormal levels of endothelial cell proliferation and apoptosis (Nagy, Chang, Shih, Dvorak, & Dvorak, 2010). Furthermore, tumours arising in cancer-prone transgenic mice are susceptible to angiogenic inhibitors (Bergers, Javaherian, Lo, Folkman, & Hanahan, 1999).

Hence, tumours must trigger proangiogenic signals, turning on angiogenesis inducers (VEGF-A) and off the inhibitors (TSP-1).

f) Metastasizing process

As the tumoral cells gain motility, like under E-cadherin (epithelial cell-cell adhesion molecule, fundamental adherents junctions formation) decreased expression, they may invade surrounding tissues, using proteolytic enzymes synthesis and extracellular release (enzymes able to break down extracellular matrix). This invasion may bring malignant cells into the body's circulatory system, the blood or lymphatic vessels. Then, they would re-cross the endothelial and basal line in order to establish secondary areas of proliferation, forming metastasis, increasing for example the E-cadherin expression (D Hanahan & Weinberg, 2000). Metastasis arise as amalgams of cancer and normal supporting cells however, while the tumour may reach the new tissue, the success of the invasion and metastasis will depend upon all hallmarks capabilities (D Hanahan & Weinberg, 2000).

In fact, this invasion depends on the nature of the metastasizing cell and the invaded tissue, in a non-random process. If the tissues produce growth factors, the invasion may be accelerated, while the presence of anti-proliferative factors, inhibitors of proteolytic enzymes and anti-angiogenesis factors bring up barriers to the invasion (Lodish et al, 2004).

Tumours may release large numbers of cells, but fewer than 1 in 10000 cells, that escaped the primary tumours, survive to originate the invasion. More than escaping and reach the blood, these cells must adhere to an endothelial cell, lining a capillary, and then migrate across or through it into the underlying tissue (Alberts, 2008).

Moreover, drastic changes occur in the cytoskeleton during tumour cell formation and metastasis, resulting from changes in gene expression. One example is the over-expression of the rhodopsin gene, increasing its activity thus leading to a metastasis stimulation (Lodish et al, 2004).

g) Emerging hallmarks

The uncontrolled chronic cell proliferation involves not only deregulated control of the proliferation but also adjustments of energy metabolism in order to fuel cell growth and division.

Even in oxygen presence, tumours can reprogram the glucose metabolism switching it to glycolysis, in a "aerobic glycolysis" state (Douglas Hanahan & Weinberg, 2011). This counterintuitive move, since this represents an approximately 18-fold lower efficiency of adenosine triphosphate (ATP) to the normal metabolism, is counterbalanced by up-regulating glucose transporters, as GLUT-1 (glucose transporter 1), resulting in higher glucose import into the cytoplasm.

Curiously, some tumours contain two subpopulations sets, one glucose-dependent and secreting lactate, and the other import the lactate, employing part of the citric to obtain energy (Weissleder & Mahmood, 2001). Furthermore, oxygenation is not necessarily static in tumours, being observed temporal and spatial fluctuations from normoxia to hypoxia. This may result from the instability and the chaotic organization of the tumour neovasculature.

Also, the immune system plays a fundamental role in the resistance and elimination of tumoral cells, blocking the formation and progression of incipient neoplasias, late-stage tumours and micrometastases (Douglas Hanahan & Weinberg, 2011). Cancers have several mechanisms that allow them to evade immunological detection and elimination. These include downregulation of cell surface proteins involved in immunogenic recognition (like MHC proteins and tumour-specific antigens), expression of other cell surface proteins that inhibit immune function (including members of the B7 family of proteins such as PD-Ll), secretion of proteins and other molecules that have immunosuppressive effects, recruitment and expansion of immunosuppressive cells such as regulatory T cells, and induction of T cell tolerance. The inflammatory effects of some of the immune mediator cells in the tumour microenvironment (especially tissue-associated macrophages and myeloid-derived suppressor cells) can also suppress T cell responses to the tumour as well as stimulate inflammation that can enhance tumour growth (Kasper et al, 2015).

The goal of biologic therapy is to manipulate the host-tumour interaction in favour of the host. Activating the immune response against tumours using immunostimulatory molecules such as interferons, 1L-2, and monoclonal antibodies have had some successes.

1.1.3. Diagnosis and Therapy

To cure or prolong the lifetime of patients, while ensuring the best possible quality of life, a huge amount of effort was done in the cancer diagnosis and treatment field. Furthermore, as cancer is easier to treat, and even cure, in the early stages, the more effective the diagnosis is, the more premature is the phase it can reach, the more its mortality can be reduced. Cancer diagnosis methods include imaging, endoscopy, biopsy, blood tests, among others, and these must be linked with an effective treatment program, provided in a sustained and equitable way, that adhere to evidence-based standards of care (World Health Organization, 2013).

A vast number of genomic alterations may be the origin of the tumour so, a variety of assays have been developed. Biomarkers, as altered DNA/gene products or from abnormal pathways, are being used as personalized treatment in techniques, as fluorescence in situ hybridization, that uses probes to confirm the presence or absence of specific DNA sequences. Polymerase chain reaction (PCR), permits amplification and analysis of target DNA regions. DNA microarray analysis measures the gene expression levels and immunocytochemistry is used to detect antigens or protein expression using a specific antibody. Flow cytometry can examine and differentiate cells based on physical and chemical properties and lastly, electron microscopy is used when specific cellular or intracellular structures need to be examined (World Health Organization, 2013).

Regardless, histological diagnosis is still essential to establish the diagnosis. However, a well-designed imaging strategy is important in the management of a patient with cancer. Imaging in oncology is used for screening, detection, diagnosis, treatment and to follow the treatment response. Image techniques enable doctors to create detailed pictures of what is going on in the body non-invasively.

Once the diagnosis is established a decision must be made regarding the most effective cancer treatment in the given socioeconomic setting treatment. This requires a careful selection of one or more of the major treatment modalities, such as surgery, radiotherapy and systemic therapy.

The selection should be based on evidence, to provide the best existing treatment given the resources available, with full knowledge that each patient and each cancer is an individual case, requiring individualized treatment. Therefore, the choice of the exact treatment or combination of treatments will depend on the patient, the disease and its stage, as well as other considerations such as performance status and comorbid conditions.

a) Surgery

Cancer surgery dates back many thousands of years, with descriptions of surgical procedures to remove breast tumours dating back as far as ancient Egypt (Edwin Smith papyrus; 1600 BC). However, what might be regarded as the first description of an oncological adequate surgical procedure dates to the 1st century AD, when Leonidas of Alexandria described a technique for stepwise incision into healthy breast tissues adjacent to a tumour, followed by serial cautery until the tumour was fully removed.

This target outcome remains the first principle of cancer surgery—to excise the tumour with a surrounding margin of healthy tissue, rather than cutting into the cancerous tissue. So, when a tumour is localized and small, sometimes, surgery or radiotherapy alone may be highly successful. Surgery plays a vital role in the prevention, diagnoses, staging, cure and palliation. Many premalignant lesions are frequently surgically removed, preventing the progression to cancer. Surgery forms the basis of therapy for early cancer, being applied for local treatment, to reduce the bulk of disease, and for removal of metastatic tumours. Although late stage cancer is generally treated by chemotherapy, surgery could offer palliation in advanced cancer.

General surgical practice has been changed since the introduction of minimally invasive techniques and better visualization techniques. Novel operative modalities have resulted in minimized surgical trauma, improvement of cosmetic outcome, and reduction of postoperative pain, hospitalization time and morbidity (Tomblyn, 2012).

b) Radiation

Radiation therapy is the administration of ionizing radiation to a cancer patient with the purpose of cure, provide palliation or as an adjuvant to surgical treatment. Radiation therapy uses high-energy radiation to reduce the size of tumours and kill cancer cells. X-ray, gamma rays and charged particles are radiation types used to the treatment. In radiotherapy, radiation may be delivered by a machine outside the body (external beam radiation therapy), from a radioactive material placed in the body near cancer (brachytherapy), or it may come by systemic administration of radioactive substances, that travel in the blood to kill cancer cells (therapy with radionuclides) (Emole, 2012).

Radiation therapy is often used in conjunction with surgery for eradication of small and limited cancers. Preoperatively, radiation therapy may be given to destroy unrecognized peripheral projections of the tumour or to reduce the size of inoperable tumours, so it can be surgically removed and be less likely to relapse. Radiation therapy given during surgery is called intraoperative radiation therapy. Alternatively, it can be given post operatively to eradicate residual. Radiation may also be used for palliation (to relieve symptoms and reduce suffering) in cases where there is no cure possible, like in cancers of the central nervous system and pathological metastasis to the bones (Emole, 2012).

b) Chemotherapy

The German chemist Paul Ehrlich, in the early 1900s, set about developing drugs to treat infectious diseases. This lead to the "chemotherapy" term, as the use of chemicals to treat disease. In the 1960s, surgery and radiotherapy dominated the field of cancer therapy until it became clear that cure rates after, ever more, radical local treatments had plateaued at about 33% due to the presence of heretofore-unappreciated micrometastases, while new data showed that combination chemotherapy could cure advanced cancers patients (D Hanahan & Weinberg, 2000).

Chemotherapy alone can be effective for a small number of cancers, such as haematological neoplasm's (leukaemia and lymphomas). As mentioned before, chemotherapeutic agents are used as primary treatment for advanced disease, but also as neoadjuvant to surgery/radiation for localized disease or as adjuvant therapy, with surgery and/or radiation (Emole, 2012).

Recent advances in genetics and molecular/cellular biology has led to an increase in the understanding of the molecular events that either initiate or sustain cancer growth. Traditional chemotherapeutic agents do not distinguish normal and cancer cells. However, these new biological agents target specific molecular pathology (pathways and aberrant genes) in cancer cells. Target therapeutics can be monoclonal antibodies or small molecules that can be used alone or in combination with other chemotherapeutics, surgery or radiation therapy (Emole, 2012).

1.2 Chronic myeloid Leukaemia

1.2.1 Introduction

Several malignant disorders, commonly named leukemia, are typified by an increased number of leukocytes building up in the blood and/or the bone marrow. Unlike other cancer types, as the cells number increase, no solid tumor formation is verified. Even though, as the normal blood cells decreases the body may have certain function compromised, such as oxygen transport, bleeding and infections control.

The increasing cells may be mostly mature, such as in chronic leukemias, or precursor cells, as in acute leukemias (Kasper et al., 2015). Furthermore, healthy blood marrow blood stem cells will mature to a myeloid or lymphoid stem cell. Myeloid will originate red blood cells, platelets or granulocytes and lymphoid stem cells will originate lymphocytes. These distinct cell types lead to the distinction between myeloid and lymphocytic leukemias.

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative hematopoietic stem-cell disorder characterized by increased proliferation of the granulocytic cell line without differentiate capacity loss.

It has an incidence of one to two cases per 100000 adults, accounting for approximately 15% of all adult leukemias, being 64 years the median age at diagnosis, with slight male predominance (Greenberg et al., 2013).

Peripheral blood shows an increased number of granulocytes and their immature precursors, including some blast cells. This disorder is one of the few cancers known to be mainly caused by a specific genetic mutation. The cytogenic aberration known as Philadelphia chromosome accounts more than 90% of the CML cases. This aberration consists of a reciprocal translocation between the long arms of chromosomes 9 and 22, resulting in a shortened chromosome 22, as shown in figure 2. This translocation relocates an oncogene called ABL to a specific breakpoint cluster region (*BCR*) in the chromosome 22. The *BCR/ABL* fusion oncogene encodes a chimeric tyrosine protein kinase, that is a novel oncoprotein of molecular weight 210 kDa referred to as $p21O^{BCR\text{-}ABL1}$. The strong tyrosine kinase activity that the chimera presents, leads to the development of CML phenotype through processes not yet fully understood. Shorter or larger BCR sequences may be fused to ABL1 resulting in variant fusion proteins, $p19O^{BCR\text{-}ABL1}$ and $p23O^{BCR\text{-}ABL1}$, being the smallest version the one which results in the worst outcome (Kasper et al., 2015).

It is known that the chimeric protein promotes growth and replication through downstream pathways such as RAS, mitogen activated protein (MAP), RAF, JUN kinase, MYC and signal transducers and activators of transcription (STAT). This influences leukemogenesis by creating a cytokine-independent cell cycle with aberrant apoptotic signals in response to cytokine withdrawal. Normal stem cells can persist and may re-emerge following effective therapy, for example with tyrosine kinases inhibitors (TKIs).

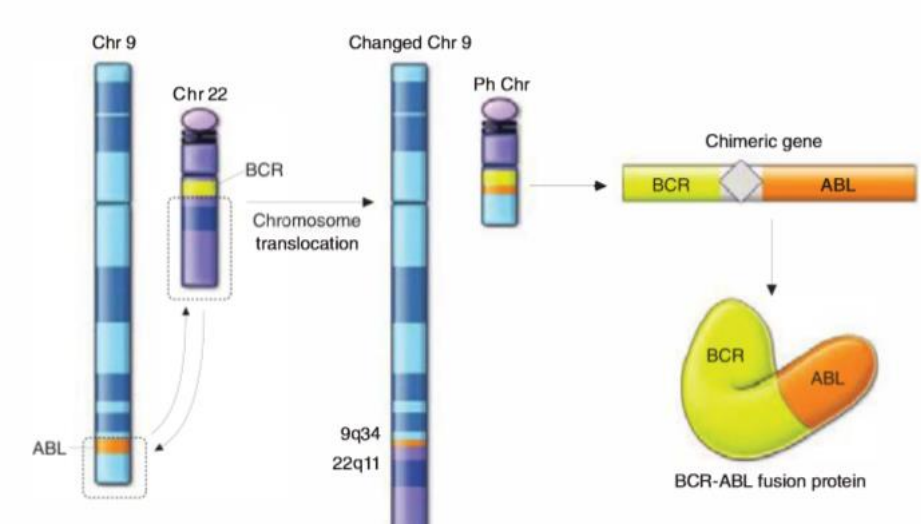


Figure 2 Philadelphia Chromosome (Ph) is derived from a reciprocaI translocation between chromosomes 9 and 22 with the breakpoint joining the sequences of the ABL oncogene with the BCR gene. The fusion of these DNA sequences allow the generation of an entirely novel fusion protein with modified function (Kasper et al., 2015).

CML progresses through three phases: chronic, accelerated, and blast. In the chronic phase of disease, mature cells proliferate. In the accelerated phase, additional cytogenetic abnormalities occur. The blast phase consists in the rapid immature cells proliferation. Approximately 85% of patients are diagnosed in the chronic phase and then progress to the accelerated and blast phases after 3-5 years (Kasper et al., 2015).

1.2.2 Incidence and Epidemiology

Important data on cancer epidemiology (like incidence, mortality, age and sex distribution, overall survival) is obtained from cancer registries covering either the entire population of a nation or selected regions with well-characterised populations (Höglund et al., 2015).

So, as previously said CML accounts for 15% of all cases of leukaemia. There is a slight male preponderance (male: female ratio 1.6:1). Furthermore, the median age at diagnosis is 55-65 years. It is uncommon in children, only 3% of patients with CML are younger than 20 years.

The annual incidence of CML is 1.5 cases per 100000 individuals. In the United States, this translates into 4500-5000 new cases per year. By extrapolation, the worldwide annual incidence of CML is about 100000 cases. The incidence of CML has not changed over several decades.

With a median survival of 6 years before 2000, the disease prevalence in the United States was 20000-30000 cases. With, tyrosine kinase therapy (TKI), the annual mortality has been reduced and the prevalence in the United States is expected to continue to increase and reach a plateau of approximately 180000 cases around 2030. The worldwide prevalence will depend on TKIs treatment penetration and their effect on reduction of worldwide annual mortality. Ideally, with full TKI treatment penetration, the worldwide prevalence should plateau at 35 times the incidence, or around 3 million patients (Kasper et al., 2015).

1.2.3 Risk factors

There are no familial associations in CML. In a study based on the Swedish Cancer Registry and Multigenerational Registry, it was found no significant familial aggregation of CML.

CML aetiology essentially unknown. No associations exist with exposures to fertilizers, insecticides, viruses, benzene or other toxins. Ionising radiation is the only established risk factor, having been linked to CML in atomic bomb survivor. Results from a recent population-based case-control study suggested a weak association between smoking and CML, but it has not yet been confirmed by other studies (Björkholm et al., 2013)

1.2.4 Diagnosis and Therapy

The availability and access to health care procedures, will impact the symptoms and clinical presentation. The proper diagnosis results in minimal symptoms and, in many cases, CML patients are asymptomatic at the time of presentation, with abnormalities identified on routine laboratory tests. However, the lack of diagnosis may lead to the presence of nonspecific symptoms as fever, night sweats, weight loss, bone pain, tiredness and even splenomegaly.

Laboratory abnormalities include leucocytosis, basophilia, eosinophilia and high platelet count. An elevates white blood cells (WBC) count, usually exceeding $25\times10^{9}/L$, with left shift is the most common feature of the disease (Greenberg et al., 2013).

Once a suspicion of CML is established, a bone marrow examination with cytogenetic and molecular testing in mandatory to confirm the diagnosis and identify the disease phase. The bone marrow is typically hypercellular with a predominance of myeloid precursors. Cytogenetic analysis will identify the presence of the Philadelphia chromosome, validating the diagnosis of CML. It is also fundamental the disease phase determination to prognosis and treatment options. At diagnosis, the large majority of the patients are in chronic phase (90% of the patients), with some patients presenting in more advanced stages, the accelerated or blast phase (Greenberg et al., 2013).

For patients diagnosed in chronic phase, the median survival is approximately 4 to 5 years without TKI therapy. Accelerated phase is a transitional phase characterized by a decrease in maturation and a median survival of 1 to 2 years. Blast phase CML is the last and most aggressive stage of the disease, resembling an acute leukaemia. The blasts can phenotypically be myeloid, lymphoid or undifferentiated. The median survival is 3 to 6 months, with lymphoid blast phase CML patients having a slightly better prognosis than those in myeloid blast phase. As the disease progresses, patients become more symptomatic, with increased anaemia, thrombocytopenia, constitutional symptoms and risk of infections (Greenberg et al., 2013).

While awaiting confirmation of the presence of the Philadelphia chromosome, hydroxyurea can be initiated for temporary control of elevated WBC counts. Once the diagnosis of CML is confirmed, TKI therapy is the mainstay treatment.

The treatment landscape has changed since the implementation of TKIs. Before 2000, allogeneic SCT was frontline therapy, when available. Allogeneic transplantation is complicated by early mortality owing to the transplantation procedure and its outcome depends on factors related to the patient (like age, stage of the disease), the donor [syngeneic (monozygotic twins) or human leukocyte antigen (HLA)-compatible allogeneic, related or unrelated], the preparative regimen, the graft-versus-host-disease (GVHD) and the posttransplantation regiment (Greenberg et al., 2013). However, transplantation is the only proven cure for CML. Less strict would be autologous transplantation, however means to select the

residual normal progenitors, which coexist with their malignant counterpart, would have to be further developed.

TKI specifically inhibit the tyrosine kinase activity of the BCR-ABL oncogene and the ABL kinase, which promotes apoptosis and cell death. Imatinib was the first TKI medication to be approved and for therapy-naive patients who were diagnosed in chronic phase and treated with imatinib, 83% achieved a complete cytogenetic remission with an overall 8-year survival rate of 85% and an 81% event free survival. Nilotinib, dasatinib and bosutinib are second generation TKIs approved for the treatment of CML. Nilotinib and dasatinib are approved for front-line therapy for CML patients and, also, as bolutinib, for those that have experienced imatinib resistance or intolerance. In fact, in newly diagnosed chronic-phase CML patients, dasatinib and nilotinib have been shown to be more potent, with better responses and outcomes than imatinib (Kantarjian et al., 2011). Ponatinib is a potent third-generation TKI currently approved for CML patients who have been resistant or intolerant to prior TKI therapy. The sixth approved agent is omacetaxine (Synribo), a protein synthesis inhibitor with presumed more selective inhibition of the synthesis of the BCR-ABL 1 oncoprotein. It is approved for the treatment of chronic- and accelerated-phase CML after failure of two or more TKIs. Table 1 shows the list of therapeutic options.

Once TKI therapy has been initiated, routine laboratory tests and follow-up are necessary to monitor for response and adverse events. A repeat bone marrow examination with cytogenetic and molecular testing is typically performed every 3 to 6 months for the first 12 months or until a cytogenetic remission has been achieved (Kasper et al., 2015).

Agent (Brand Name)	Approved Indications	Dose Schedule	Notable Toxicities
Imatinib mesylate (Gleevec)	All phases	400 mg daily	See text
Dasatinib (Sprycel)	All phases	First-line: 100 mg daily Salvage: 140 mg daily	Myelosuppression; pleural and pericardial effusions; pulmonary hypertension
Nilotinib (Tasigna)	All phases except blastic phase	First-line: 300 mg twice daily Salvage: 400 mg twice daily	Diabetes; vasoocclusive disease; pancreatitis
Bosutinib (Bosulif)	All phases except frontline	500 mg daily	Diarrhea
Ponatinib (Iclusig)	All phases except frontline	45 mg daily (may consider lower starting doses in the future, e.g., 30 mg daily)	Skin rashes, pancreatitis; vasoocclusive disease (10–20%)
Omacetaxine mepesuccinate (Synribo)	Failure ≥2 tyrosine kinase inhibitors	1.25 mg/m² subcutaneously twice daily for 14 days for induction; 7 days of maintenance every month	Myelosuppression

Table 1 Medical Therapeutic Options in Chronic Myeloid Leukaemia (Imatinib will be further explored below), (Kasper et al., 2015).

1.2.5 Guidelines

There are some guidelines for the management of CML. In fact, National Comprehensive Cancer Network (NCCN), European Society of Medical Oncology (ESMO) and European LeukemiaNet (ELN) have issued guidelines that go through the tree phases of this disease. For chronic phase the guidelines

recommend any of three tyrosine kinase inhibitors (TKIs) (imatinib, nilotinib, or dasatinib) as first-line treatment for CML and in case of intolerance, other TKI or bosutinib may be used. As second-line treatment all three TKIs can be used, possibly varying the doses depending on the chosen agent. Hematopoietic stem cell transplantation (HSCT) should be considered in the case of failure of two TKIs (Copelan, 2006).

For accelerated-phase CML it is recommended a TKI like imatinib, nilotinib, dasatinib or bosutinib, being the base of the choice prior therapy and/or BCR-ABL kinase domain mutation status. The recommended doses are generally higher for this phase. If there is resistance and/or intolerance to two or more TKIs occurs, omacetaxine or HSCT can be considered, with the latter having concerns about the age of the patient.

Lastly, for blast phase it is recommended HSCT, preferably after response to induction therapy. Furthermore, patients in lymphoid blast phase can be treated with acute lymphoblastic leukemia (ALL) induction chemotherapy regimens in combination with a TKI. Patients in myeloid blast crisis can be treated with acute myeloid leukemia (AML) induction chemotherapy regimens in combination with a TKI.

To perform monitoring, NCCN, ELN and ESMO guidelines recommend bone marrow cytogenetics and quantitative reverse transcription polymerase chain reaction (QPCR) as monitoring response to tyrosine kinase inhibitor (TKI) therapy tests. However, the recommendations regarding response to first-line treatment vary between the three guidelines,

In general, BCR-ABL1 transcripts should be lower than 10% at three months. European LeukemiaNet, is stricter and at six months requires Philadelphia chromosome positive (Ph+) at least equal to 35% and BCR-ABL1 lower than 1%, while NCCN keeps the 10% BCR-ABL1 levels. At twelve months, BCR-ABL1 should not be higher than 0,1% (Baccarani et al., 2012; Pallera et al., 2016).

1.2.6 Imatinib

Imatinib was approved for medical use by Food & Drug Administration (FDA) in 2001. Imatinib is a 2-phenyl amino pyrimidine derivative that functions as a specific inhibitor of a number of tyrosine kinase enzymes. It occupies the tyrosine kinase (*TK)* active site, leading to a decrease in its activity. Therefore, is used in the treatment of multiple cancers, most notably Philadelphia chromosome-positive (Ph +) chronic myelogenous leukemia. Like all tyrosine kinase inhibitors, imatinib works by inhibiting the tyrosine kinase enzyme. Because the BCR–Abl tyrosine kinase enzyme exists only in cancer cells and not in healthy cells, imatinib works as a form of targeted therapy. In this regard, imatinib was one of the first cancer therapies to show the potential for such targeted action and is often cited as a paradigm for research in cancer therapeutics – the “magical bulllet”.

The active sites of tyrosine kinases have a binding site for ATP. The enzymatic activity catalyzed by a tyrosine kinase is the transfer of the terminal phosphate from ATP to tyrosine residues on its substrates, a process known as protein tyrosine phosphorylation. Imatinib works by binding close to the ATP binding site of *bcr-abl*, locking it in a closed or self-inhibited conformation, and therefore inhibiting the enzyme activity of the protein semi-competitively (Kasper et al., 2015). This fact explains why many BCR-ABL mutations can cause resistance to imatinib by shifting its equilibrium toward the open or active conformation.

Imatinib is rapidly absorbed when orally taken and have high bioavailability (98% reaches the bloodstream). Imatinib metabolism occurs in the liver and is mediated by several isozymes of the cytochrome P450 system, including CYP3A4 and, to a lesser extent, CYP1A2, CYP2D6, CYP2C9, and CYP2C19. The main metabolite, *N*-demethylated piperazine derivative, is also active. The major route of elimination is in the bile and feces, only a small portion of the drug is excreted in the urine. Most of imatinib is eliminated as metabolites, only 25% is eliminated unchanged. The half-lives of imatinib and its main metabolite are 18 h and 40 h, respectively. It blocks the activity of Abelson cytoplasmic tyrosine kinase (ABL), c-Kit and the platelet-derived growth factor receptor (PDGFR). As an inhibitor of PDGFR, imatinib mesylate appears to have utility in the treatment of a variety of dermatological diseases. Imatinib has been reported to be an effective treatment for mast cell diseases, hypereosinophilic syndrome, and dermatofibrosarcoma protuberans (Carrol et al., 1997).

The only known contraindication to imatinib is hypersensitivity (Becker & Jordan, 2011), and as imatinib is highly plasma protein-bound, is hard to proceed to its extraction (Savi et al., 2018). However, as there are many *TK* enzymes in the body, and imatinib is specific for the *TK* domain in b *abl* (the Abelson proto-oncogene), c-kit and PDGF-R (platelet-derived growth factor receptor), side effects may occur.

Clinical effectiveness of imatinib mesylate in cancer treatment can be compromised by its off-target toxicity, as cardiotoxicity. Cardiovascular complications are included among the systemic effects of TKI-based therapeutic strategies. Cardiac anatomy in exposed rats showed a dose-dependent, restrictive type of remodelling and depressed hemodynamic performance in the absence of remarkable myocardial fibrosis. The effects on rat and human cardiovascular progenitor cells (CPCs) were also assessed. Rat CPC depletion was induced, with reduced growth and increased cell death. Similar effects were observed in CPCs isolated from human hearts (Savi et al., 2018).

Furthermore, side effects, including nausea, vomiting, diarrhea, headaches, bruising or bleeding are also a possibility (Benny et al., 2009, Kasper et al, 2015), and cutaneous reactions in patients treated with imatinib are common, occurring in 7%–88.9% of patients. They include superficial edema, pigmentary changes, pruritus and, in rare cases bullous reactions like pseudoporphyria, porphyria and Stevens–Johnson syndrome (Carroll et al., 1997). Overall, cutaneous reactions to imatinib seem to be dose-

dependent including severe to life-threatening skin manifestations in a higher dose starting at 600 mg/d, suggesting a dose-related pharmacologic effect of the drug rather than hypersensitivity (Muhl, Ehrchen, & Metze, 2017).

There are cases of a chronic myeloid leukaemia patients with an inadequate response to imatinib due to concurrent phenytoin administration. The observed interindividual variability relies on pharmacokinetic and pharmacodynamic variations that can be attributed to genetic, environmental and pathological causes, among others. Thus, the presence of drug-to-drug interactions (DDIs) between imatinib and concurrent medication may also play a relevant role (Milojkovic & Apperley, 2009).

Despite imatinib by itself being a remarkably effective drug, some mechanisms of resistance have been identified. Experimental evidences suggest that poor intracellular accumulation of the drug and emergence of mutated clones primarily give rise to the resistance against such drugs (Bouchet et al., 2013; Branford et al., 2003). Moreover, drug influx/efflux ratio appears to be one of the key determinants of therapeutic outcomes (Mukherjee et al., 2018).

Recent strategies to address drug resistance are focused on combinatorial therapy with TKIs, conjugating nanoparticles with the TKIs entrapping the drug inside liposomes or the use of anti-BCR-ABL siRNA. However, liposomal entrapment of drugs is still a challenging issue (Gurudevan et al., 2013). In addition, drug dissociation step inside the treated cells is critical to obtain optimal response.

1.3 Pancreatic Cancer

1.3.1 Introduction

Pancreatic cancer is classified according to which part of pancreas is affected by it. Exocrine, the digestive substances production part is affected, or endocrine if the hormones production part is the affected. As the exocrine pancreas makes up 95% of the pancreas, there is a corresponding higher arising cancer numbers. Endocrine cancer types are rarer and named accordingly to the type of hormone produced (insulinoma: insulin-producing cell; glucagonomas: glucagon-producing cell; gastrinomas: gastrin-producing cell, among others) (Kasper et al., 2015).

Pancreatic cancer is associated with a number of well-defined molecular hallmarks. The four genes most commonly mutated or inactivated in pancreatic cancer are KRAS (predominantly codon 12, in 60-75% of pancreatic cancers), the tumour-suppressor genes p16 (deleted in 95% of tumours), p53 (inactivated or mutated in 50-70% of tumours), and SMAD4 (deleted in 55% of them) (Maitra et al., 2009).

The pancreatic cancer precursor lesion pancreatic intraepithelial neoplasia (PanIN) acquires these genetic abnormalities in a progressive manner associated with increasing dysplasia. Initial KRAS mutations are followed by p1610ss and finally p53 and SMAD4 alterations. SMAD4 gene inactivation is associated with a pattern of widespread metastatic disease in advanced-stage patients and poorer

survival in patients with surgically resected pancreatic adenocarcinoma. Some germline mutations, that may be inherited (16% of pancreatic cancers) are associated with a significantly increased risk of pancreatic cancer and other cancers. For example STKll gene (PeutzJeghers syndrome carries a 132-fold increased lifetime risk of pancreatic cancer above the general population), BRCA2 (increased risk of breast, ovarian, and pancreatic cancer), p16/CDKN2A (familial atypical multiple mole melanoma, carries an increased risk of melanoma and pancreatic cancer), PALB2 (which confers an increased risk of breast and pancreatic cancer), hMLHl and MSH2 (Lynch syndrome, which carries an increased risk of colon and pancreatic cancer) and ATM (ataxia-telangiectasia carrying an increased risk of breast cancer, lymphoma, and pancreatic cancer). Familial pancreatitis and an increased risk of pancreatic cancer are associated with mutations of the PRSSl (serine protease 1) gene. However, in most familial pancreatic syndromes the genetic cause remains unexplained (Maitra et al., 2009).

Surrounding pancreatic adenocarcinoma, the desmoplastic stroma functions as a mechanical barrier to chemotherapy and secretes compounds essential for tumour progression and metastasis. Key mediators of these functions include the activated pancreatic stellate cell and the glycoprotein SPARC (secreted protein acidic and rich in cysteine), which is expressed in 80% of pancreatic ductal adenocarcinomas. The metastasization process typically reaches regional lymph nodes, the liver and sometimes the lungs. Surrounding visceral organs, such as duodenum and stomach, may also be invaded (Kasper et al., 2015).

1.3.2 Risk factors

Cigarette smoking is the most common recognized risk factors for pancreatic cancer and may account over 20% of all pancreatic cancers. Others include obesity, high alcohol consumption, history of pancreatitis, family history of pancreatic cancer, and possibly selected dietary factors (Bosetti et al., 2012). Diabetes also appears to be a risk factor, however it may also occur in association with pancreatic cancer, possibly confounding this interpretation (Kasper et al., 2015).

1.3.3 Incidence and Epidemiology

Although accounts for only about 3% of all cancers in the United States, pancreatic cancer is the fourth leading cause of cancer deaths at United States, being responsible for 7% of all cancer-related deaths in both men and women. Approximately 75% of all pancreatic carcinomas occur within the head or neck of the pancreas, 15-20% occur in the body of the pancreas, and 5-10% occur in the tail (Ilic & Ilic, 2016).

In the absence of predisposing conditions, such as familial pancreatic cancer and chronic pancreatitis, pancreatic cancer is unusual in persons younger than 45 years. After age 50 years, the frequency of pancreatic cancer increases linearly.

1.3.4 Diagnosis and Treatments

Since the initial symptoms of pancreatic cancer are often quite nonspecific and subtle, pancreatic cancer is notoriously difficult to diagnose in its early stages. Patients typically report the gradual onset of nonspecific symptoms such as anorexia (weight lose), malaise, nausea, fatigue and pain. At the time of diagnosis, 85-90% of patients have inoperable or metastatic disease, which is reflected in the 5-year survival rate of only 6% for all stages combined. An improved 5-year survival of up to 24% may be achieved when the tumour is detected at an early stage and when complete surgical resection is accomplished (llic et al., 2016).

Potentially useful tests in patients with suspected pancreatic cancer include the following:

- CBC count;
- Hepatobiliary tests: Patients with obstructive jaundice show significant elevations in bilirubin (conjugated and total), ALP, GGT, and, to a lesser extent, AST and ALT;
- Serum amylase and/or lipase levels: Elevated in less than 50% of patients with resectable pancreatic cancers and in only 25% of patients with unresectable tumours;
- Tumour markers such as CA 19-9 antigen and CEA: 75-85% have elevated CA 19-9 levels; 40-45% have elevated CEA levels;

Furthermore, imaging studies, like CT scanning, transcutaneous ultrasonography, endoscopic ultrasonography, magnetic resonance imaging, endoscopic retrograde cholangiopancreatography and positron emission tomography scanning may aid in the diagnosis of pancreatic cancer.

The treatment will be dependent on the diagnosis. Localized nonmetastatic disease is potentially suitable for surgical resection. Approximately 30% of patients have R1 resection (microscopic residual disease) following surgery. Those who undergo R0 resection (no microscopic or macroscopic residual tumour) and who receive adjuvant treatment have the best chance of cure, with an estimated median survival of 20-23 months and a 5-year survival of approximately 20%. Outcomes are more favourable in patients with small (<3 cm), well-differentiated tumours and Iymph node-negative disease. Adjuvant chemotherapy comprising six cycles of gemcitabine is common practice (Kasper et al., 2015).

Surgery is the primary mode of treatment for pancreatic cancer. However, an important role exists for chemotherapy and/or radiation therapy. Antineoplastic agents and combinations of agents used in managing pancreatic carcinoma. Approximately 30% of patients present with locally advanced, unresectable, but nonmetastatic pancreatic carcinoma. Gemcitabine treatment leads to a survival time of approximately 9 months. Patients who respond to chemotherapy or who achieve stable disease after 3-6 months of gemcitabine have frequently been offered consolidation radiotherapy.

Approximately 60% of patients with pancreatic cancer present metastasis. Gemcitabine was the standard treatment with a median survival of 6 months and a 1-year survival rate of only 20%. The addition of

nab-paclitaxel (an albumin bound nanoparticle formulation of paclitaxel) to gemcitabine results in an improvement. There is a search for new combinations in order to improve the results *in vitro* and in clinical trials (Giordano et al, 2017).

1.3.5 Guidelines Summary

There are some guidelines for pancreatic cancer. In fact, U.S. Preventive Services Task Force (USPSTF), American Academy of Family Physicians (AAFP) and International Cancer of the Pancreas Screening (CAPS) Consortium issued guidelines.

The USPSTF found no evidence that screening for pancreatic cancer is effective in reducing mortality and recommends against routine screening in asymptomatic adults using abdominal palpation, ultrasonography, or serologic markers. The AAFP guidelines concur with the USPSTF recommendation. In 2012, the International CAPS Consortium, a panel of 49 multidisciplinary experts, released consensus guidelines for pancreatic cancer screening. While also recommending against routine screening in the general population, the members recommended screening with endoscopic ultrasound and/or magnetic resonance imaging/magnetic resonance cholangiopancreatography for some high-risk groups, namely individuals with two or more blood relatives with pancreatic cancer, *p16*, *PALB2*, or *BRCA2* mutations carriers with a first-degree relative with pancreatic cancer, individuals with Peutz-Jeghers syndrome and also Lynch syndrome patients with a first-degree relative with pancreatic cancer (Canto et al., 2012).

Both the European Society of Medical Oncology (ESMO) and the National Comprehensive Cancer Network (NCCN) guidelines for diagnosis and treatment of pancreatic cancer recommend the measurement of serum CA 19-9 levels after surgery and before adjuvant therapy to guide treatment and follow up.

1.4 Nanotechnology

1.4.1 Introduction

In 1959, Richard Feynman brought the possibility of writing the entire 24 volumes of the British encyclopaedia at the head of a pin. The nanoworld was open and, in 1989, Don Eigler wrote the IBM logotype only using 35 xenon atoms. This also marked the nanotechnology concept birth. Nanotechnology is the application of science and technology to the study and control of matter at the molecular level (less than 1 µM, usually between 1-100nm) and the production of materials, structures, systems and devices in the nanoscale range.

This range presents several advantages as the materials have different chemical, physical and biological properties. Mechanical, electrical, optical and magnetic properties, can be explored in order to obtain

the desired outcome (Khan, Gupta, Verma, & Nandi, 2015; Shvets; J. Zhang et al., 2016).Furthermore, these materials also have a larger surface area, resulting in a higher area/volume ratio. Thus, more surface area is available to interact with the surrounding material (Khan et al., 2015).

To sum-up, the nanometer, that measures one-billionth of a meter, brought the biological size. In fact, many biological structures range at the nanometer scale, as DNA molecule at 2.5 nm, and protein molecule at 1–20 nm. Tryptophan, the biggest amino acid is at 1.2 nm (Brancolini, Kokh, Calzolai, Wade, & Corni, 2012). Therefore, nanotechnology can be applied beyond fields such as electronics, information technology and material developments, having also great relevance in the biomedical areas and medicine. Nanobiotechnology represents a specific category of nanotechnology based on biological molecules and processes that involve the creation of systems and devices, at the nanolevel, to examine or control biological processes (Leo, Vandelli, Cameroni, & Forni, 1997). In medicine the main applications can be divided in diagnosis, imaging and drug delivery.

Varying with the size, chemical composition, surface structure, solubility, shape and aggregation, the physiochemical properties of the nanomaterials may present distinctive characteristics, being of malleable usability and presenting different advantages depending on the formulation (Mao et al., 2014; Nasr, Nafee, Saad, & Kazem, 2014).

Despite this, nanotoxicity may be an alarming problem to population. The novel properties raise concerns about the interactions and biological systems effects (Otto, Otto, & de Villiers, 2015;). Furthermore, some nanoparticles may travel throughout the body, deposit in target organs, penetrate cell membranes and, in a completely unknown way, communicate with the ambient and possibly leading to injurious responses (E. S. Kim, Ahn, Chung, & Kim, 2013).

Therefore, it is important to be proper ascertain of the oxidative stress, inflammation, immune reactivity, foreign body formation, protein denaturation as well DNA and membrane damage (Fakruddin, Hossain, & Afroz, 2012; D Hanahan & Weinberg, 2000; E. S. Kim et al., 2013). As nanoworld is a not yet fully understood and known world, novel mechanisms of toxicity may emerge, resulting in new tests requirement. However, even if nanomaterials do not introduce new pathologies, with the various tests outcomes normally reduced to a limited number such as inflammation, apoptosis, necrosis, fibrosis, hypertrophy, metaplasia and carcinogenesis, there could be novel mechanisms of injury that would require special tools, assays, and approaches to assess their toxicity (Tietze et al., 2015). Furthermore, there is a strong likelihood that biological activity and toxicity will depend on physicochemical characteristics that are not usually considered in toxicity screening studies.

1.4.2 Nanotechnology in cancer

Despite significant efforts toward research and treatment development, cancer continues to be a major health world problem. Problem that is only further enhanced by the heterogeneous nature of the disease. Nanotechnology has evolved as a technology with applications to medicine and the potential to improve clinical outcomes, with its application to cancer garnering much attention. Through the generation of novel nanoscale devices and therapeutic platforms, nanotechnologies have emerged as innovative approaches that enable the detection and diagnosis of cancer at its earliest stages, and the delivery of anticancer drugs directly to tumours.

Improvements have been made to chemotherapies, but drugs to reach the tumour site at effective doses and are often associated with high systemic toxicities and poor pharmacokinetics.

Nanotechnology advantages are numerous and include the selective targeting and delivery of anticancer agents to tumour tissues and devices for early cancer detection and imaging systems. Thus, these approaches can be used to enhance tumour regression by delivering multiple types of therapeutics, or they can be used to monitor therapeutic efficacy by combining therapeutic and imaging agents in a single multifunctional platform through either encapsulating or conjugating existing chemotherapeutics to their surfaces (W. Y. Kim & Lee, 2009;Rizvi & Saleh, 2017)

Furthermore, with optimized design, the timing or site of drug release can be controlled by material composition or via a triggered event, such as light exposure, ultrasound, or change in pH (Otto et al., 2015).

A variety of ligands can be used to target tumour cells or microenvironment, such as aptamers, antibodies, growth factors and small molecules. Several studies have shown that active targeting and receptor-mediated internalization are necessary for genetic therapies and can increase the anticancer activity of the associated drug, with significant potential for translation to the clinic. However, targeted nanotechnology-based platforms have yet to fully make the transition from concept to clinical translation. There are multiple reasons for this, but primarily stem from concerns regarding consistency in reproducing targeted nanoparticles with the same ligand density and activity, reducing nonspecific biomolecule interactions and optimizing the ligand density with surface marker expression on tumour cells, which may be highly variable (Vakser, 2014, Nel, Xia, Mädler, & Li, 2006; Park, 2012).

In addition to their role in drug delivery, nanomaterials have been used to detect and diagnose early-stage cancers, identify metastases noninvasively and guide tumour removal during surgery. For image guided surgery, targeted nanoparticles can provide molecular imaging functionality through site- or activity-specific localization. Iron oxide nanoparticles provide a sensitive, low-toxicity alternative to standard MRI contrast agents, such as injected gadolinium (Gunn, Paranji, & Zhang, 2009).

Nanoparticles demonstrated the capability to reduce side effects, allowing different and adjustable drug doses, however additional results that strongly support improved survival rates are still needed (Khan et al., 2015).

1.4.3 Nanocarriers

Nanocarriers can be fabricated from a variety of materials with the advantage of possibly being used for controlled release of drugs. Nanocarriers can be programmed to degrade within a certain time and will be recycled or exit the body through urine or feces, proving a treatment space and temporal control, and potential low toxicity (Kratz, 2008).

The potential routes of administration include gastrointestinal tract, skin, lung, and systemic administration. However, the interactions of the nanocarriers with cells, body fluids, and proteins play a key role in the biological effects and ability to distribute throughout the body. Binding to proteins may generate complexes possibly leading to agglomeration and blood vessels obstruction. More mobile complexes that can enter tissue sites, normally inaccessible, may also appear which can be both an advantage (facilitating for example the entrance in cancer tissues by drugs) or a disadvantage since it can bring unexpected toxicity and side effects (Elsadek & Kratz, 2012).Throughout the journey, several obstacles and barriers may appear, possibly resulting in elimination or sequester, in a non-efficient drug transport.

Endocytic events are regulated by clathrin-coated pits and caveolae (caveolin protein covered plasma membrane indentations, as well as scavenger receptors, like SCARA5. These events are dependent on the nanocarrier size, but surface coatings need to be also considered. It is known that some coats like albumin, lecithin, polysorbital 80 or peptide attachments can enhance nanoparticle uptake into cells. (Donoso et al., 2018; Treuel, Jiang, & Nienhaus, 2013).

Three key elements of a toxicity screening strategy should include physicochemical characterization of nanocarrier, in vitro assays (cellular and noncellular), and in vivo studies (Sharma, Madhunapantula, & Robertson, 2012).

1.4.4 Protein Nanocarriers

As previously stated, a vast number of materials have been described for nanoparticles fabrication, however biocompatibility is a fundamental requirement. To the material is required an appropriated host response depending on the application.

Protein as GRAS (generally regarded as safe) drug delivery systems due to their exceptional characteristics, namely biodegradability, nonantigenecity, high nutritional value, abundant renewable resources and extraordinary binding capacity to various drugs. Proteins lead to less opsonization by the reticuloendothelial system (RES) through an aqueous steric barrier in addition to their excellent functional properties including emulsification, gelation, foaming and water binding capacity (Swami et al., 2015; Kratz, F.,2008). Moreover, they can be easily prepared and scaled up during manufacture. Thus, there is an increasing development in protein nanocarriers.

Protein nanocarriers, due to their multiple functional groups present in the primary sequences of polypeptides, can be exploited to create various interactions with compounds, forming three-dimensional networks with numerous possibilities of reversible binding of active molecules, protecting them (D. Chow, Nunalee, Lim, Simnick, & Chilkoti, 2008).

Furthermore, proteins are recyclable. In fact, they are naturally metabolized by digestive enzymes being the result of this hydrolysis bioactive peptides that may exert several physiological effects or just serve as new building blocks. Despite the absorbability and biodegradation advantage, depending on the protein type and origin different grades of toxicity may result. This is an important aspect to be inferred (Agrahari, Agrahari, & Mitra, 2016).

Several proteins were already explored to function as nanocarrier. Examples such as gelatin, a denatured protein obtained from collagen, has a long history of safe use in pharmaceuticals, cosmetics and food products being considerate as GRAS material. Since it is denatured it has low antigenicity and its functional groups are accessible to various chemical modifications particularly useful for targeting delivery systems development. Along its many application in the biomedical field, it has been reported successfully as delivery system for many purposes such as antitumoral, antiviral and microbial (García, 2018).

Collagen itself has also been widely used, due to its biocompatibility, in fact it accounts for about 20-30% of total body proteins, low antigenicity and biodegradability. Another example of a biocompatible, non-toxic, non-immunogenic, biodegradable and metabolized into innocuous degradation products is albumin. Therefore, over the past decades, albumin has appeared as a successful and versatile drug carrier (Ulery, Nair, & Laurencin, 2011).

To conclude, proteins are a viable carrier that can exhibit high loading capacity, with multiple binding sites and a variety of loading mechanisms (electrostatic and hydrophobic interactions as well covalent

bonding). Targeting and further surface properties improvements are allowed, due to its innumerous functional groups present at its surface, resulting in a better drug transport, lower side effects in an efficient controlled drug release. Furthermore, they are much less expensive compared to synthetic polymer. However, some complications do exist. Proteins may form heterogenous mixtures, thus producing heterogenous nanoparticle sizes distribution and batch-to-batch variation (Mullen & Banaszak Holl, 2011), hindering industrial applications. Moreover, due to protein hydrophilicity and rapid solubilization in aqueous environments there is a certain inability to obtain a controlled drug release.

A better understanding of the mechanism of action of protein vehicles and interactions protein-drug is fundamental to further optimization and to reach the ideal nanocarrier for drug and even gene delivery.

1.5 Ferritin

1.5.1 Introduction

Fundamental to the body, iron participates in phenomenon like electron transfer, nitrogen fixation, ribonucleotide reduction (essential to DNA synthesis) and, one of the most known iron biological employments, the heme oxygen transport, storage and release. Furthermore, is also required for critical enzymatic functions, vital to several cellular processes as cell cycle, electron transport, reductive conversion of ribonucleotides to deoxyribonucleotides, among others (Theil, 1987).

However, iron can also be toxic. Its potential to electron donation to superoxide radical formation and its participation in the hydroxyl radicals' generation via Fenton reaction

$(\mathrm{Fe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(III)} + \mathrm{OH^-} + \mathrm{OH}\cdot$, shows how much iron stats may dramatically affect the generation of oxygen, ferryl and nitrogen radicals, indicating its potential toxicity. In fact, most of the iron cellular systems toxicity is attributable to its capacity to participate in the generation of such reactive species, which can directly damage DNA, lipids, and proteins, leading to profound cellular toxicity. Therefore, it is imperial to maintain the iron balance with exquisite care (Gunshin et al., 1997).

Found in most cell types and highly conserved (Theil, 1987), ferritin is one of the major non-heme iron binding proteins. In most tissues, ferritin proteins are mainly present in the cytosol, nucleus and mitochondria.

Ferritin manage, captures and storage the intracellular labile iron pool (Kakhlon, Gruenbaum, & Cabantchik, 2001; Picard et al., 1996) playing a key role in maintaining iron homeostasis. To further prove ferritin importance in iron homeostasis and, as well, iron toxicity, homozygous murine knockouts of ferritin are lethal (Ferreira et al., 2000).

The iron storage capability presented by ferritin is related to its structure. Ferritin molecules are made of two components: a protein shell, named apoferritin, that forms a hollow cage of 12 nm of external and 8 nm of internal diameter and allows the inside presence of the second component, a microcrystalline core of approximate composition [Fe(III)O·OH] containing, at most, about 4500 iron atoms along with variable amounts of phosphate.

The apoferritin shell is composed by twenty-four monomeric subunits, in a mixture of ferritin H chain (FHC) and ferritin L chain (FLC) (Theil, 1987). Furthermore, as FHC weights ~21kDa, while FLC ~19kDa (Boyd, Vecoli, Belcher, Jain, & Drysdale, 1985), they are also referred as heavy and light ferritin respectively (Theil, 1987) and ferritin H chain was initially found on heart while L chain on liver. The FHC/FLC ration varies from specie to specie and even from organ to organ, variation that is also observed during processes like the inflammatory one (P Arosio, Yokota, & Drysdale, 1976; L. Li et al., 2010).

Not so common as intracellular, extracellular ferritins are found in fluids, such as serum as well as synovial and cerebrospinal fluids (CSF) (Wang, Knovich, Coffman, Torti, & Torti, 2010). Are predominantly composed of L-chains, which have a lower iron content, and, although several studies have reported that serum ferritin might arise from the secretion of hepatocytes, macrophages, and Kupffer cells (Wang et al., 2010), the source and even the function of secreted ferritin is still not totally understood (Fan, Gao, & Yan, 2013).

Regardless, not only does the FHC/FLC ratio varies but also the serum protein concentration may also vary. In fact, serum ferritin is elevated in many malignancies (Paolo Arosio, Ingrassia, & Cavadini, 2009; Wang et al., 2010) and even in cancer. Furthermore, it may also occur a shift in ferritin composition to a more H-rich species (Hann, Stahlhut, & Millman, 1984) and, for example, in malignant histiocytosis serum ferritin is mostly constituted by ferritin H. Despite these changes underlying mechanisms not being fully understood, in neuroblastoma, an increase in serum ferritin has been directly linked to ferritin secretion by tumour itself (Kabat & Rohan, 2007). Regardless of which, no studies to date have demonstrated that ferritin contributes more to the cancer than merely being a serum marker for cancer presence. However, iron overload and iron homeostasis disruption may contribute to carcinogenesis (Wang et al., 2010).

Further studies on ferritin secretor cells and receptors are required to better understand ferritin pathways (Meyron-Holtz, Moshe-Belizowski, & Cohen, 2011). However, transferrin receptor 1 (TfR1) binds to H ferritin that enters endosomes and lysosomes, the latter possibly facilitating iron release due to low pH and the unique ferritin structure, despite physiologic pH rigidness when brought into acidic conditions it is observed a nanocage break down into subunits (Kabat & Rohan, 2007; Wang et al., 2010).

1.5.2 Ferritin as a nanocarrier

Naturally present in the organism, ferritin is biocompatible, non-immunogenic and binds specifically to receptors such as TfR1, overexpressed in diseases like cancer. Being smaller than many other drug carriers, ferritin may allow a longer circulation half-time, also related to the non-immunogenicity, and a better tumour accumulation rate. It has a unique structure as previously stated, however one important fact is that when the pH is turned back to physiologic conditions, the subunits will reassemble the ferritin nanocage in a nearly intact fashion, via the disassembly–reassembly route, figure 3. This can also be used to achieve hybrid nanocages and the uniform size of the ferritin cage origins monodispersed nanoparticles. Moreover, the nanocage surface can be easily modified, either genetic or chemically.

The ferritin nanoparticles have several applications in drug deliver, catalysis and imaging, among others Quantum dots can be prepared inside the ferritin cage for effective growth inhibition of colorectal carcinoma cells via generation of ROS. PbS-ferritin QDs were found to have NIR photoluminescence properties which are useful in combination with anticancer activity for cancer diagnostics, imaging and treatment.

The ferritin cage also provides suitable coordination environments for metal complexes for the generation of composites used in various applications. Organometallic $[Pd(allyl)Cl]_2$ and $[Rh(norbornadiene)Cl]_2$ complexes immobilization into the ferritin cage to promote catalytic C–C coupling and polymerization (Abe, Maity, & Ueno, 2016).

Several different metal complexes have functionalized the ferritin cage, with the aim of controlling cellular functions, developing imaging agents and for drug delivery. Cisplatin and its analogues have been incorporated into the ferritin cage via the assembly/disassembly route. Pt particles were synthesized inside the ferritin cage by incubating K_2PtCl_4 in ammonia buffer (pH 8.5), enhancing the loading efficiency.

Receptor mediated endocytosis is thought to be responsible for the higher uptake of ferritin loaded drugs. The cytotoxicity was found to be less than that of naked drugs due to limited release from the cage. However, the results indicate that ferritin has potential to be developed as a promising carrier for anticancer drugs.

For magnetic resonance imaging it is possible to incorporate into ferritin Gd (III)–chelate complexes. The cage can accommodate about 10 paramagnetic units, providing long term stability and low leaching rate. It is expected that the interaction between the protein interior cage and free exchangeable protons is responsible for the higher relaxivity.

Organic molecules, like doxorubicin (Dox) were incorporated into the ferritin cage to improve pharmacokinetics, activity and selectivity and, despite high loading, the ferritin cage structure was retained.

The preparation of nanocomposites or metal functionalization using ferritin cage can be achieved in a single step that does not require additional targeting or stabilizing ligands. Thus, the ferritin cage is a promising nanocarrier for developing new biomaterials which are expected to provide the basis for new therapeutic and imaging applications (Abe et al., 2016).

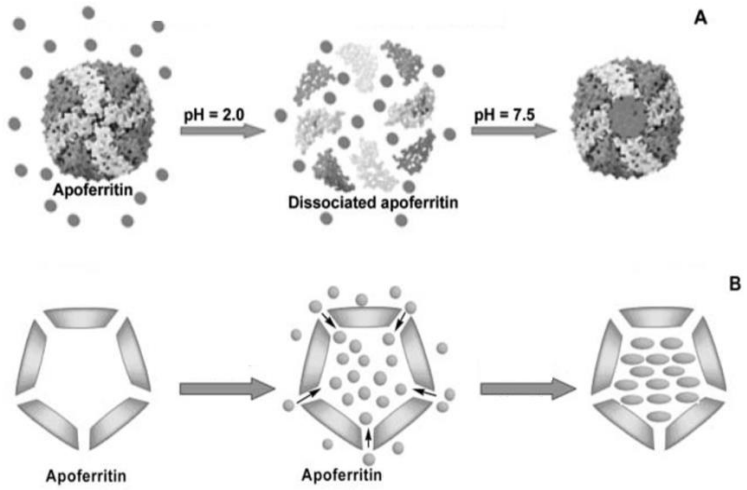


Figure 3 Ferritin compound incorporation via disassembly/reassembly (A) and direct diffusion (B) (Guo Z. et al.,2007)

2. Preparation and characterization of Ferritin – Drug complexes

2.1. Materials and Methods

2.1.1. Reagents

Horse spleen ferritin (F4503), Imatinib mesylate (CDS022105) and Cisplatin (P4394) were purchased from Sigma and used without further purification. All other reagents were of analytical grades.

2.1.2. Circular Dichroism experiment

CD spectra were recorded by a J-815 (JASCO) spectrometer set at 20°C. Ferritin protein samples at 0.1 mg/ml diluted in citrate-phosphate buffer under specific pH conditions (8.0, 7.0, 6.0, 4.0, 3.0, 2.6, 2.0). Spectra recorded in a 1 mm cell, from 260 to 190 nm, at 50 nm/s, with a scanning interval of 2s, data-pitch 0.1 nm and 8 accumulations per measurement. Spectra smoothed using the Savitzky–Golay algorithm and corrected for the blank sample.

The same analysis was performed using ferritin samples at 0,1 mg/mL under different methanol concentrations (60-80%) at different time points.

2.1.3. Ferritin-Drug Complexes preparation

a) Disassociation/Reassociation method (Imatinib encapsulation)

Imatinib loading into the ferritin cage was carried out based on Guo Z. et al.,2007, with slight alterations. Briefly, ferritin at 0.1 mg/mL was dissolved in 8M urea and gently mixed for 30min at room temperature to ensure the complete dissociation of the ferritin. Imatinib Mesylate was added to a final concentration of 20 μM and incubated for 15 minutes at room temperature. The mixture was dialyzed against gradient concentrations of urea buffer (2 and 0 M), supplemented with 20 μM Imatinib, at 4ºC for several hours.

b) Diffusion Method (cisplatin)

Ferritin solution, 66 μM, was dissolved in 8M urea and gently mixed for 30min. Dialysis against analytical grade II water was peformed to promote iron removal and apoferritin cage reassociation. Then, the apoferritin solution was mixed with cisplatin aqueous solutions (0 and 0,9% NaCl), 3333 μM, at a ratio of 1:100 mol/mol. Diffusion into ferritin cage promoted with a 48 hours incubation in the dark under slight agitation. Then, KCl was added (0.1M) and the solution kept in the dark for 24 hours, under agitation.

c) In situ generation method (cisplatin based NMOF)

The *in situ* generation method was performed based on Wenbin Lin et al., 2014, with slight modifications. Firstly, c,c,t-$Pt(NH)_2Cl_2(OH)_2$ was synthetized. A mixture of cis-$Pt(NH_3)_2Cl_2$, a.k.a. cisplatin, (0.025 g) and H2O2 (30 wt%, 0,14 mL) in H2O (1,125 mL, pH 7) was heated at 70 ºC with vigorous magnetic stirring for 5 hours in the dark. The heat was removed and stirring was continued overnight. After concentrating the mixture to ~100 µL, the product was allowed to precipitate at 4 ºC over several hours. The product was collected via vacuum filtration, washed with ice cold H_2O and vacuum dried.

Then, a mixture of c,c,t$Pt(NH_3)2Cl_2(OH)_2$ (1.00 mg) and succinic anhydride (1.20 mg) in dimethyl sulfoxide (DMSO) (3.00 µL) was heated at 70 ºC in the dark for 24 hours with magnetic stirring. The solvent was subsequently removed via lyophilization. The pale yellow product (DSCP) was recrystallized from acetone at -20 ºC, isolated via vacuum filtration, and washed with ice cold acetone.

Lastly, TbCl3 (4.5mg) and DSCP (6mg) were mixed into an apoferritin solution at 2.5 µM. Incorporation into the apoferritin cage was promoted by incubation in the dark for 2 hours. Then, methanol was slowly added until reaching 30% methanol. After two hours, methanol was removed by dialysis (FCT1). Urea (8M) was added to part of the solution and incubated for half an hour. Dialysis was performed to remove the urea and the protein renatured (FCT2).

2.1.4. Ferritin-Drug Complexes characterization

a) TEM analysis

For transmission electron microscopy analysis, 10 µL of samples were mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 1 minute. The liquid in excess was removed with filter paper.

Visualization was carried out on a JEOL JEM 1400 TEM at 120kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan). A brief uranyl staining was also performed in some samples.

For EDS analysis, the sections were mounted on nickel grids and a beryllium holder (EM-21150, Jeol Ltd.) was used. An X-Max 80 mm2 (Oxford Instruments, Bucks, England) operated at 120 kV was coupled to the microscope, at the HEMS / i3S of the University of Porto.

b) ICP-MS analysis

Three samples of ferritin-cisplatin solutions, resulting from the diffusion method, at 22µM were dialysed against an aqueous solution cisplatin free, for 4 hours. Cisplatin in the first sample was obtain from a

0,9% NaCl solution, the second was dissolved from a 0,9% NaCl to water for 4 hours and the third was a H_20 cisplatin solution. The dialyzed and the dialysis solution were collected, and further dissolved two and five hundred times in 3% HCl: 1% HNO3 solution, respectively. Platinum concentration was measured on an iCAP Q ICP-MS (Thermo Scientific, Germany).

2.1.5. Release Quantification assay

Imatinib-loaded ferritin was dialyzed at room temperature against varying pH conditions (0,1M NaAc pH5, 0,1M citrate-phosphate buffer pH6,5, PBS 10x pH 7,4 and 0,1M Tris-HCl pH8,5). 1 mL aliquots were removed at different incubation time points (and replaced with fresh buffer) and the release was determined spectrophotometrically at 290nm. As a positive control, free imatinib mesylate at 0.5 mg/mL was dialyzed under pH 7.4.

2.2. Results

2.2.1. Protein stability under encapsulation conditions

Dissociation of ferritin can be accomplished either by denaturation or acidification. The effect of these phenomena in the structure of ferritin was analyzed by CD spectroscopy and are shown in Figure 4. It is observed that acidification to pH=2 does not present clear ellipticity curve band differences, figure 4A. However, it is noticeable that at pH 4, one negative band is lost.

Urea mediated denaturation presented an increasing pronounced effect in the secondary structure of the protein with the increase of its concentration, figures 4B, 4C. The measure range decrease on urea is due to the HT increase, that should be preferentially under 700V (figure S1, annexes).

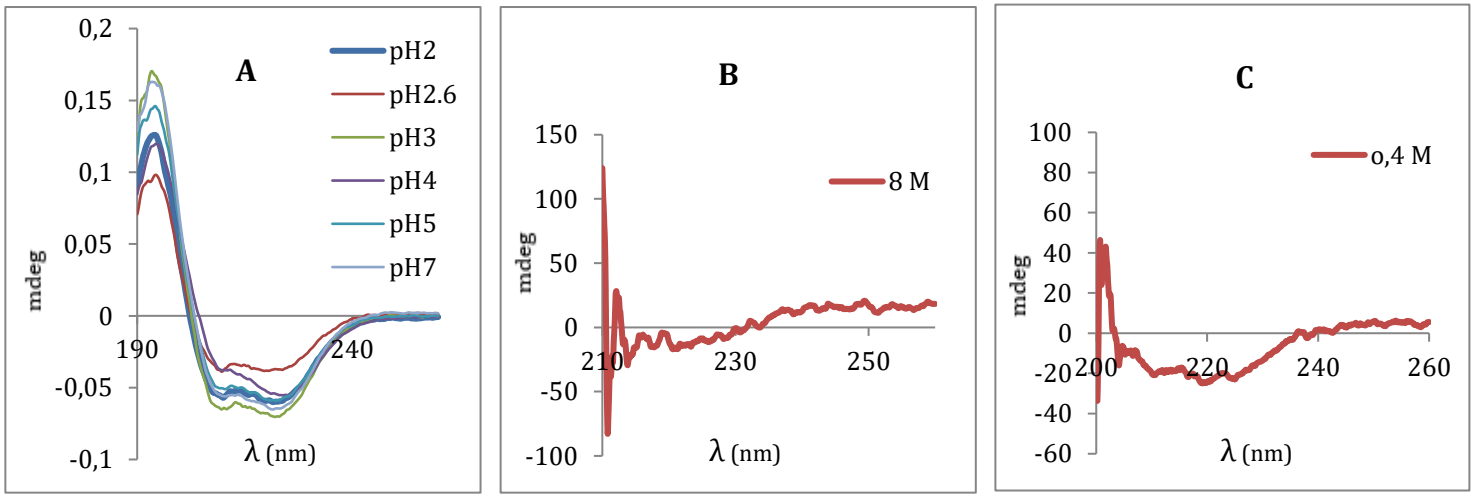


Figure 4 Horse spleen ferritin maintains its secondary structure at different citrate-phosphate buffer pHs (7, 5, 4, 3, 2.6, 2) and under different urea conditions (B-8M; C 0,4M)

Methanol effect on ferritin cage was also tested. Figure 5B shows that at 80% methanol concentration, the typical ferritin ellipticity curve was lost, figure 5A. At 70% and 60% methanol, ferritin cage was maintained initially, figures 5C, 5D. However, after 0.5 hours the effect on 70% methanol was measured and showed an undefined curve. The same effect was observed at 5hours on 60% methanol.

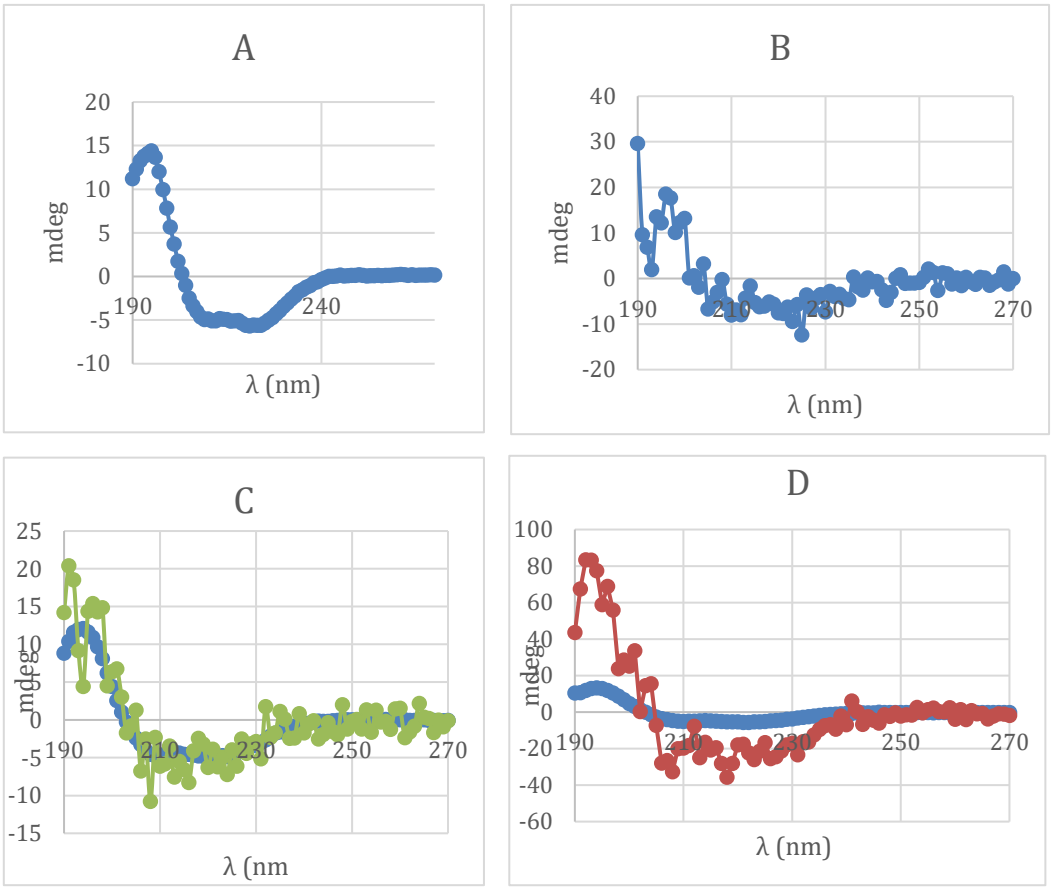


Figure 5 Methanol CD experiment ferritin at 0,1 mg/mL. A) Ferritin in Water; B) Ferritin in 80% Methanol; C) Ferritin in 70% Methanol; D) Ferritin in 60% Methanol. – Blue line represents CD analysis immediately after adding methanol; – Green line represents CD analysis after 5 hours of adding methanol; – Red line represents CD analysis after 0.5 hours of adding methanol.

2.2.2. Imatinib-release quantification

The imatinib release profile at different pHs is shown in figure 6. The release is progressive and pH dependent. After 24 hours at pH 7.5 around 36% is released, at pH 6.5 around 42% and at pH 5 around 66%. However, in the early experiment stages, the different conditions did not presented differences. Free imatinib release experiment, figure 7, presented an initial release burst in the first hour, followed up by a stabilized phase.

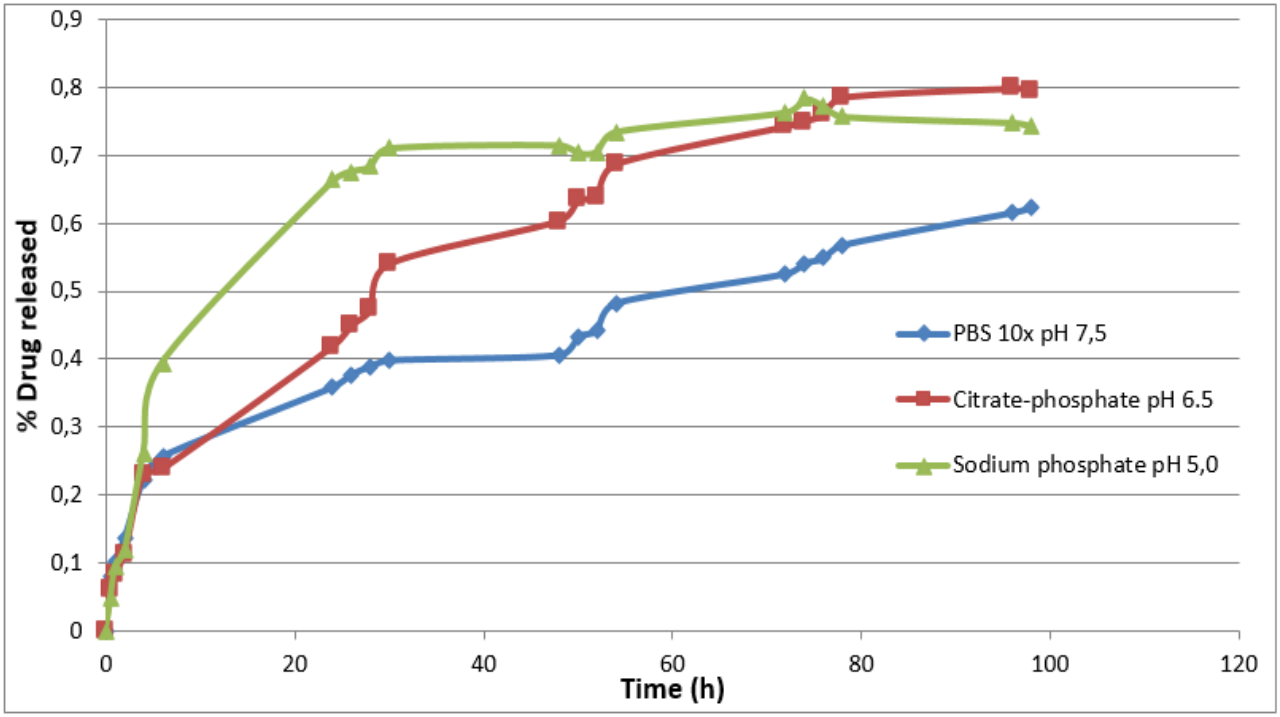


Figure 6 It was promoted the encapsulation of imatinib on ferritin nanocage and then the release by dialysis under different pH conditions (-- pH 7,5; -- pH 6,5; -- pH 5). No representation of pH 8,5 since it was not sure the initially present quantity (some volume losses occurred during the procedures).

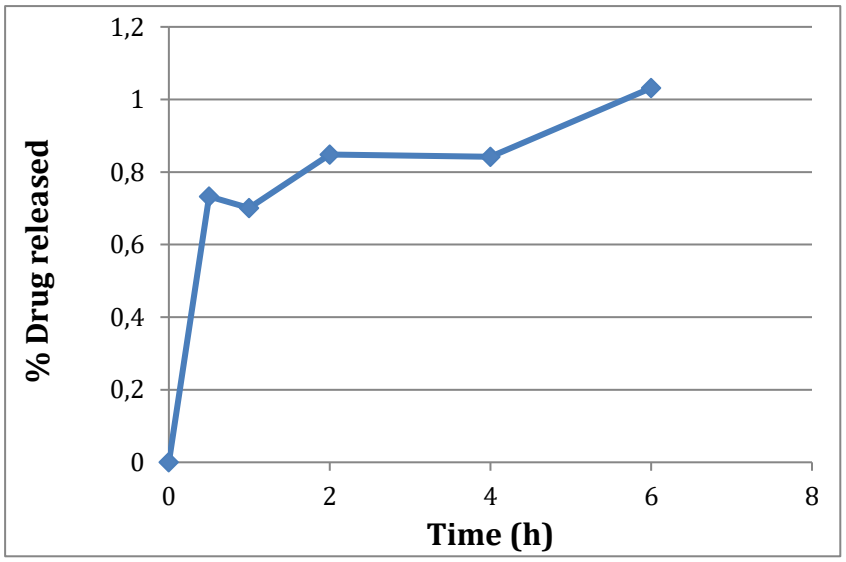


Figure 7 Free imatinib was dialysed under PBS10x under pH 7,5.

2.2.3. Ferritin-Cisplatin Complex Characterization

a) TEM analysis

TEM analysis, figure 8, showed clear organized electrodense points, which may correspond to ferritin incorporation signal. Free cisplatin showed no organization, with big agglomerates found.

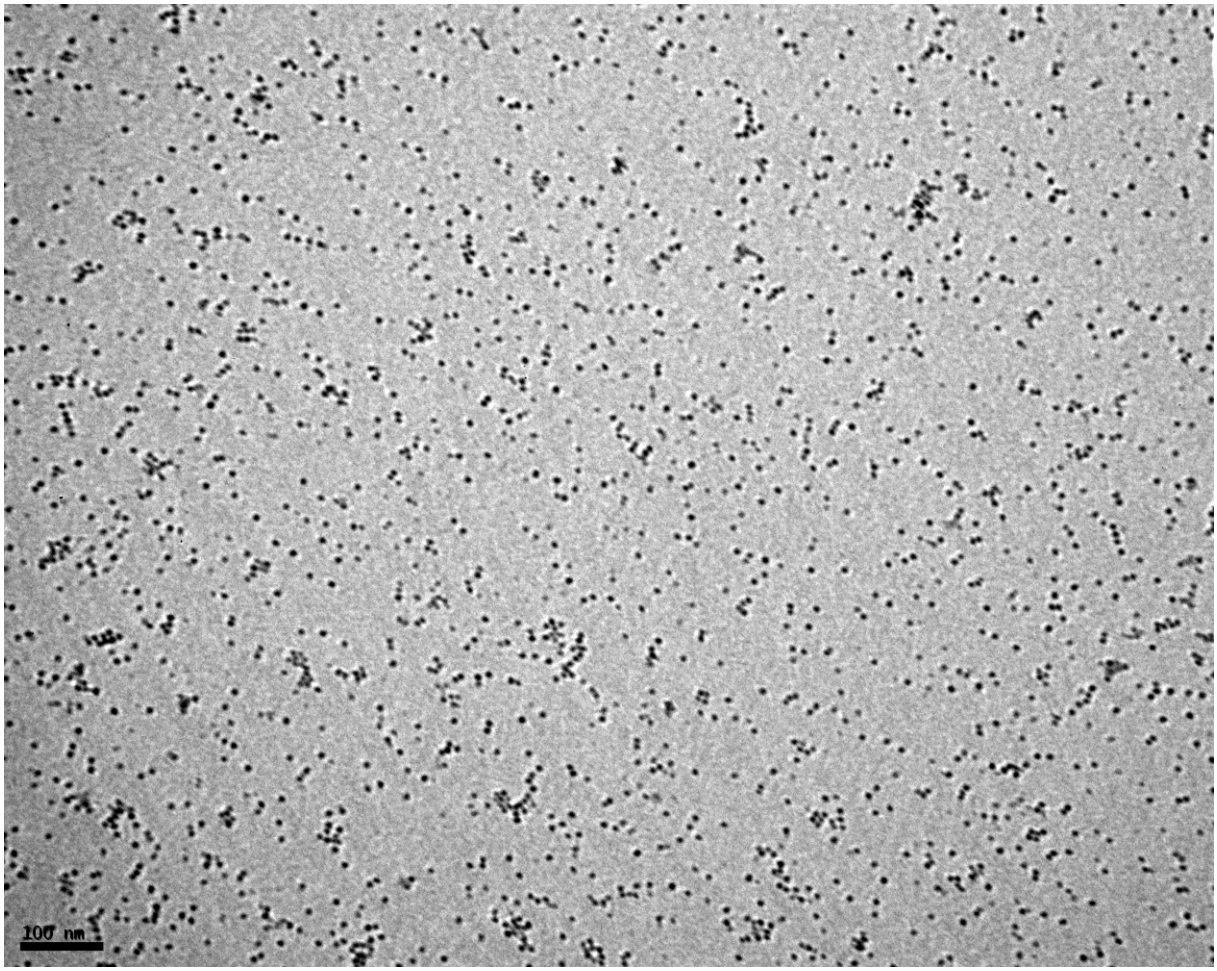


Figure 8 TEM analysis. Ferritin-Cisplatin conjugates observed at 10000x.

b) ICP analysis

Ferritin-Cisplatin conjugates solution was dialyzed, and the Pt concentrations measured, figure 9A. Cisplatin, previously dissolved in NaCl (FC1), presented the highest Pt concentration of the dialyzed solution,5150ng/L. Cisplatin dissolved in Water (FC3) presented the lowest, 809,5 ng/L. Cisplatin that was transferred to water (FC2) was at 4941,5 ng/L. Moreover, Figure 9C shows that, in the dialysis solution, FC3 has the highest concentration, 655,28 ng/L and FC1 the lowest 14,23 ng/L. FC2 was in the middle with 23,36 ng/L.

After adding the respective Pt quantities, present in both the dialyzed and the dialysis solutions, no significant differences were found (FC1 1,04 ng, FC2 1,01 ng and FC3 1,06 ng).

This implies, that cisplatin previously dissolved in water, and positively charged, may lead to the lowest encapsulation efficacy. In fact, contrasting with the 98,6% presented by FC1, and almost presented by FC2 (97,7%), FC3 only presented only 38,2% encapsulation efficacy.

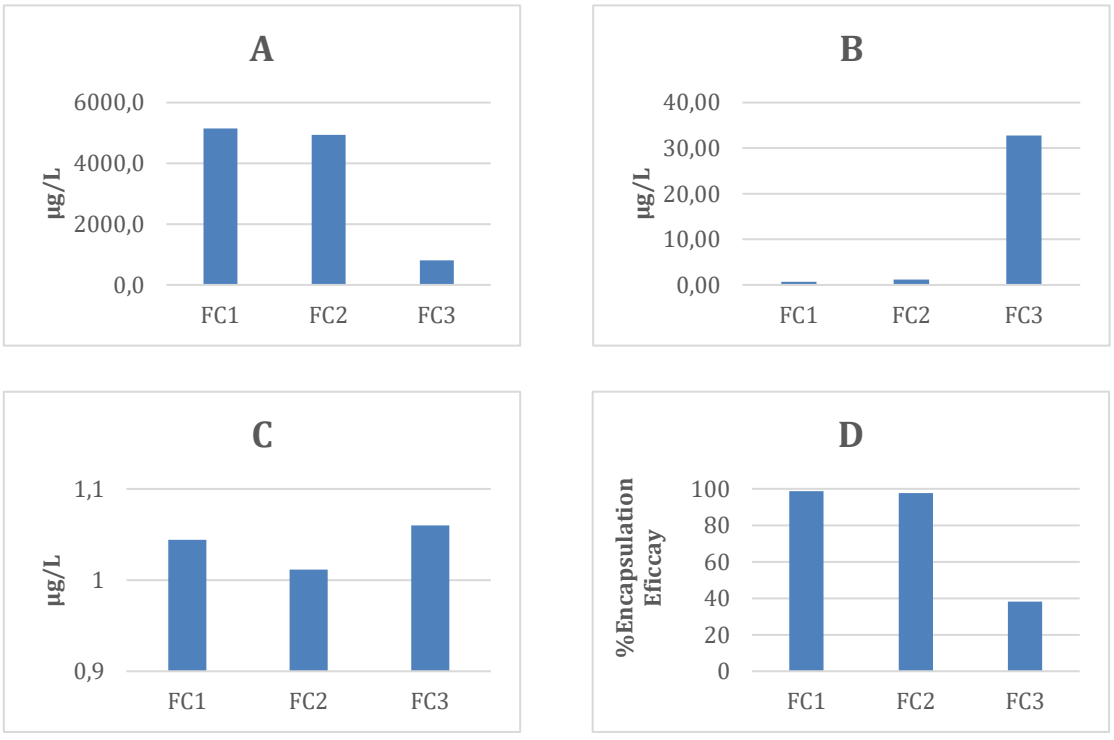


Figure 9 Figure that represents the Ferritin-Cisplatin in NaCL (FC1), the Ferritin-Cisplatin in NaCL/H2O (FC2) and the Ferritin-Cisplatin in H20 (FC3) Pt concentrations in A) Dialysed Solution and B) Dialysis Solution. It is represented the absolute Pt quantities in the same conditions C) and the encapsulation efficacy D).

2.2.4. Cisplatin based NMOF-Ferritin Complex Characterization

The cisplatin based NMOF-Ferritin complex was analysed recurring to transmission electron microscopy. The solution containing this complex was optically divided in three phases before measure. It was then mixed, and homogenized and tree samples were observed. The first sample was stained with uranyl, figure 10 A, and showed apoferritin without any compound electrodense incorporated compound. The apoferritin presented a normal, as reported structure (Guo Z. et al.,2007). The second one, figure 10 B, showed large compounds formation. The third sample, figure 10 C, showed small electrodense compounds, possibly ferritin incorporated. EDS analysis, figure 11, on the samples, showed terbium, cisplatin and iron presence.

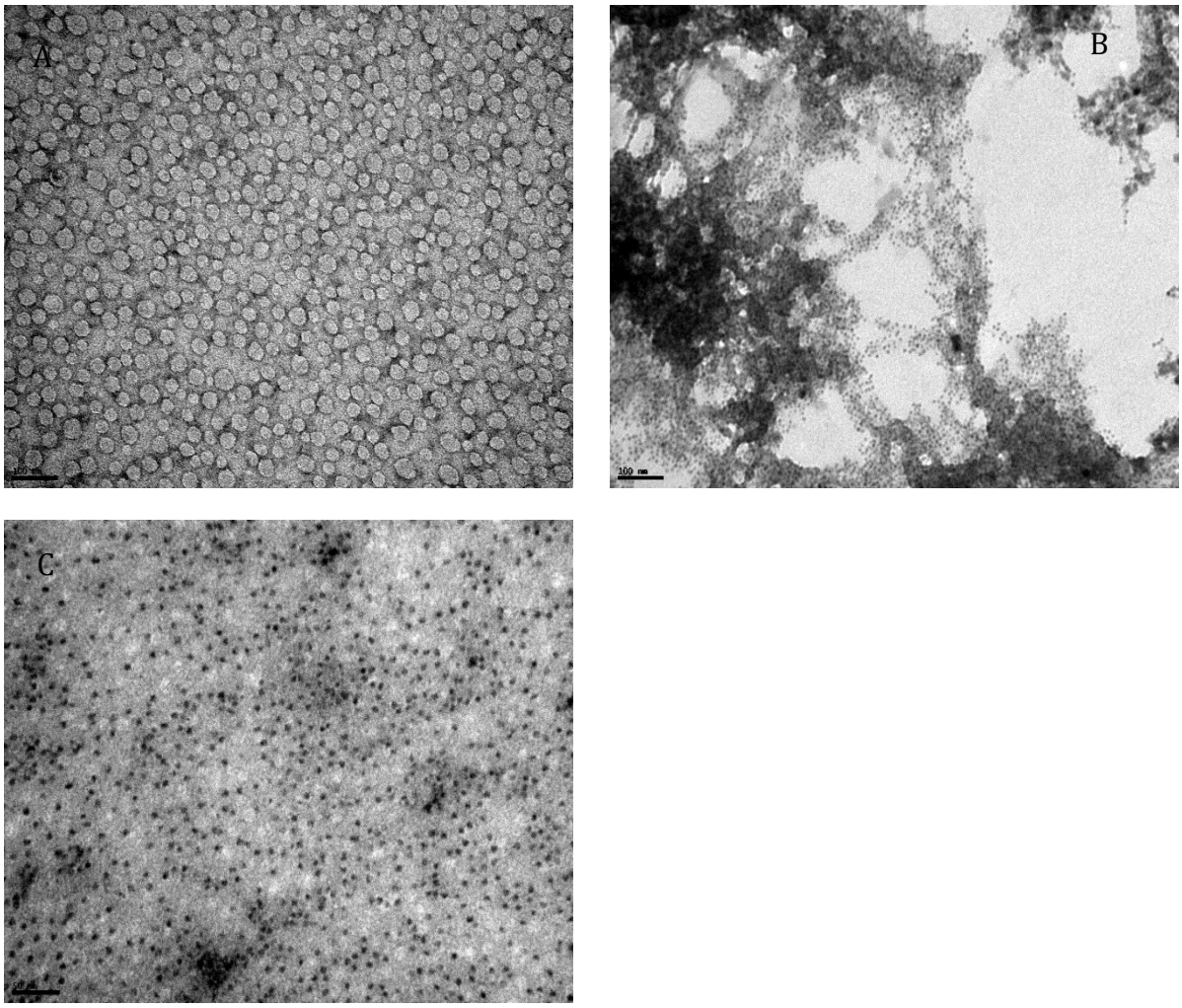
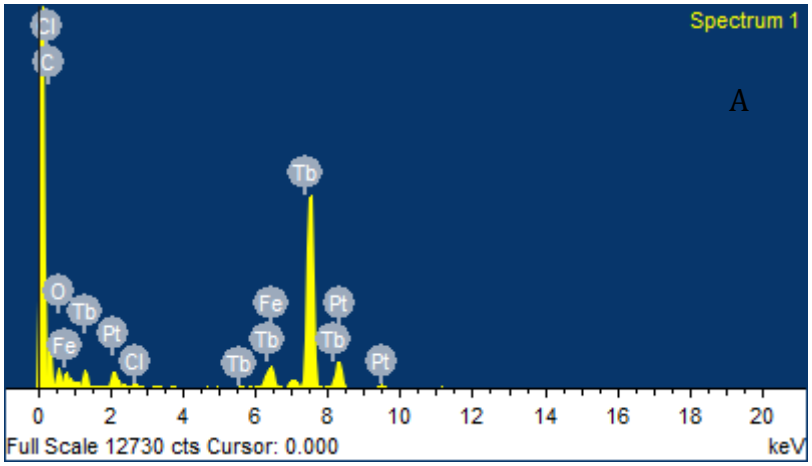
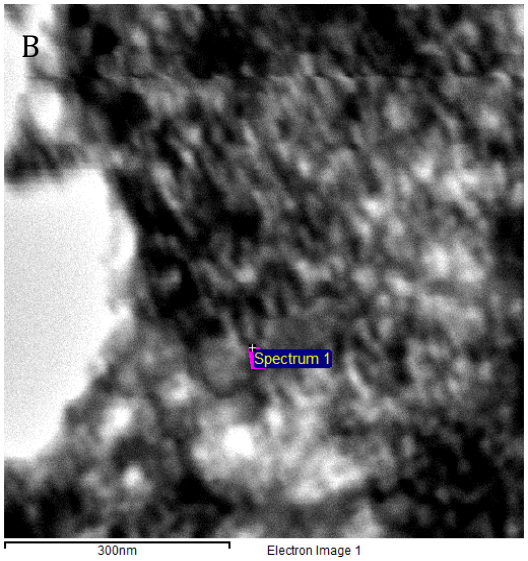


Figure 10 Ferritin-NMOF compounds TEM analysis. A) Ferritin (120000x) uranyl stained; B) Aggregates (120000x) no stain; C) Ferritin + Compound (250000x)no stain.





C

Element	Weight%	Atomic%
C	5.82	22.68
O	14.44	42.24
Cl	1.87	2.47
Fe	19.61	16.43
Tb	40.40	11.90
Pt	17.85	4.28
Totals	100.00	

Figure 11 Ferritin-NMOF conjugation energy dispersive spectrometry (EDS) analysis. A) X-ray spectrum; B) Zone visualization; C) Elemental relative atomic proportions

3. Citotoxicity

3.1. Materials and Methods

K562 cell line was purchased from European Collection of Cell Cultures (ECACC) and Panc1 cell line from American Type Culture Collection (ATCC). All other reagents and materials were sterilized before coming into contact with the cells. Cells were routinely monitored by PCR for possible mycoplasma contamination (Venor ® GeM Advance Mycoplasma Detection Kit, Minerva).

3.1.1. Cell Culture

K562 cells are grown in suspension. Cells were routinely maintained at 5×10^5 cells/mL in RPMI medium supplemented with 10% fetal bovine serum (FBS) in a humid atmosphere containing 5% CO2 at 37°C. K562 culture medium was renewed twice a week and viability, given by equation 1, was maintained at least at 90%. Healthy cells were counted on a neubauer chamber, and their concentration was determined using trypan blue reagent (this reagent enters in cells that have cell membrane damage and thus staining death cells with blue colour). Since live cells are excluded from staining, this staining method is also described as a dye exclusion method. Once a week, centrifugation at 800 rpm for 6 minutes was performed and the supernatant discarded. Dead cells were thus discarded in the supernatant, while living cells remained as a pellet which was then resuspended in fresh medium.

Equation 1: $Cells\ viability\ = \frac{Healthy\ cells}{Total\ Cells}$

Panc1 cells are adherent cells. They were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) in a humid atmosphere containing 5% CO2 at 37°C. Panc1 culture medium was renewed twice a week. When confluency was observed, firstly the medium was removed, cells washed with PBS to eliminate death cells, and trypsin added. Trypsin is a serine protease from the PA clan superfamily, found in the digestive system of many vertebrates. It has the ability to cleave peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline, in a process known as trypsinization. This process leads to the loss of the adhesion cellular property and cells enter in a suspension mode. Cellular concentration was maintained, at 5×10^5 cells/mL, and cells were allowed to grow.

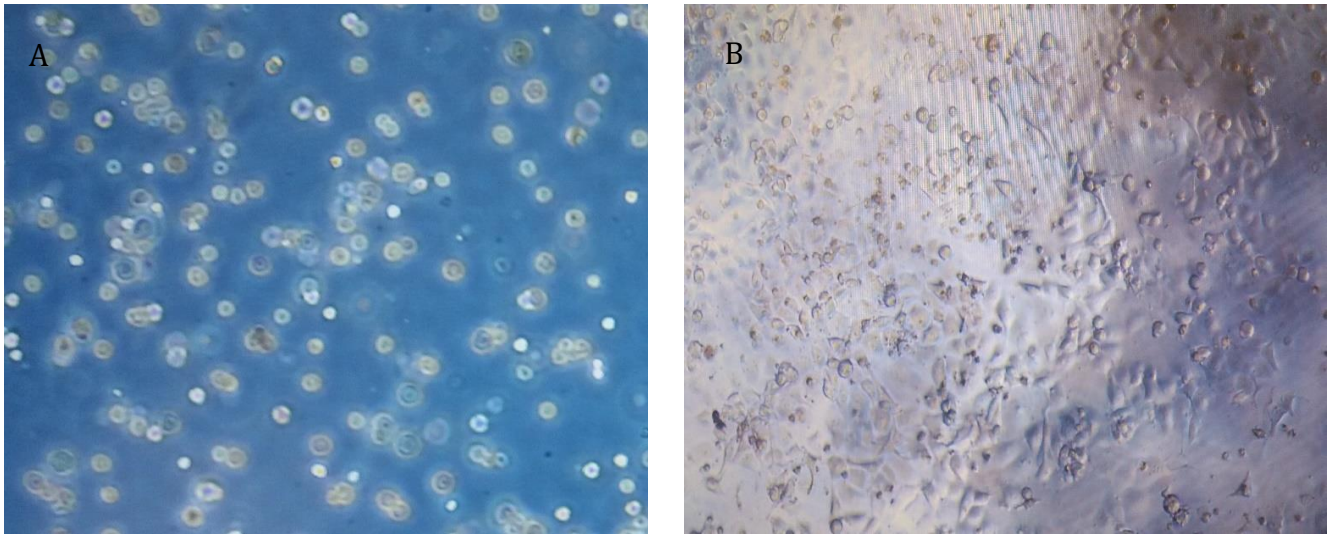


Figure 12 K562(A) and Panc1(B)cells in culture with RPMI supplemented with 10% FBS

3.1.2. Evaluation of metabolic effects of Ferritin conjugations using Resazurin assay

K562 and PANC1 cells were seeded in a 96 well plate at concentration of 5×10^4 cells/mL, and incubated for 24 hours in a humid atmosphere containing 5% CO2 at 37°C. The cell concentration was determined recurring at trypan blue using a neubauer chamber. The compounds: Imatinib, Cisplatin and both with Ferritin, as well as Ferritin alone (used as control) were then added to the cells. Several concentrations were tested: 2μM for imatinib loaded conjugation and 100μM for cisplatin (higher concentrations) and from them several serial dilutions were used. Compounds without the presence of cells were also used to take out the background. Cells were allowed to continue to grow for further 48 hours at the same incubation conditions. In the end, resazurin was added, at a final concentration of 40μM for 3.5 hours. Fluorescence was measured in a microplate reader at 530nm emission and 590 nm remission.

3.1.3. Panc1 Cell Line treated with Ferritin conjugations TEM analysis

After resazurin assay, Panc1 cells were washed and trypsin was added. After 10 min of incubation, the samples were collected.

Then, samples were fixed in 2,5% glutaraldehyde (Electron Microscopy sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in cacodylate Buffer 0.1M (pH 7.4), dehydrated and embedded in Epon resin (TAAB, Berks, England).

Ultrathin sections (40–60 nm thickness) were prepared on a RMC Ultramicrotome (PowerTome, USA) using diamond knives (DDK, Wilmington, DE, USA).

The sections were mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 15 min each, and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W Tokyo, Japan at the HEMS / IBMC - Institute for Molecular and Cell Biology (IBMC) of the University of Porto.

3.2. Results

3.2.1. Ferritin-Imatinib Metabolic Effects

K562 tumoral cells were incubated under increasing imatinib concentrations, ranging from 0.125 to 2 µM. No significative difference was found between free imatinib (I) and imatinib loaded apoferritin (FI) at 1:1250 mol/mol ferritin:imatinib ratio, figure S2, annexes. Despite that, a slight improve was observed at the lowest imatinib concentrations.

Apoferritin imatinib loaded in a 1:100 mol/mol ratio, showed a significant difference between conditions. As seen in Figure 13, FI conjugation demonstrated a higher metabolic effects, with lower metabolic activity at all concentrations when compared with free imatinib. Table 2 presents the IC_{50} of these conjugations. Ferritin-imatinib presented a 0,125 µM and free imatinib a 0,245 µM IC_{50}.

Both conditions presented no ferritin toxic effect.

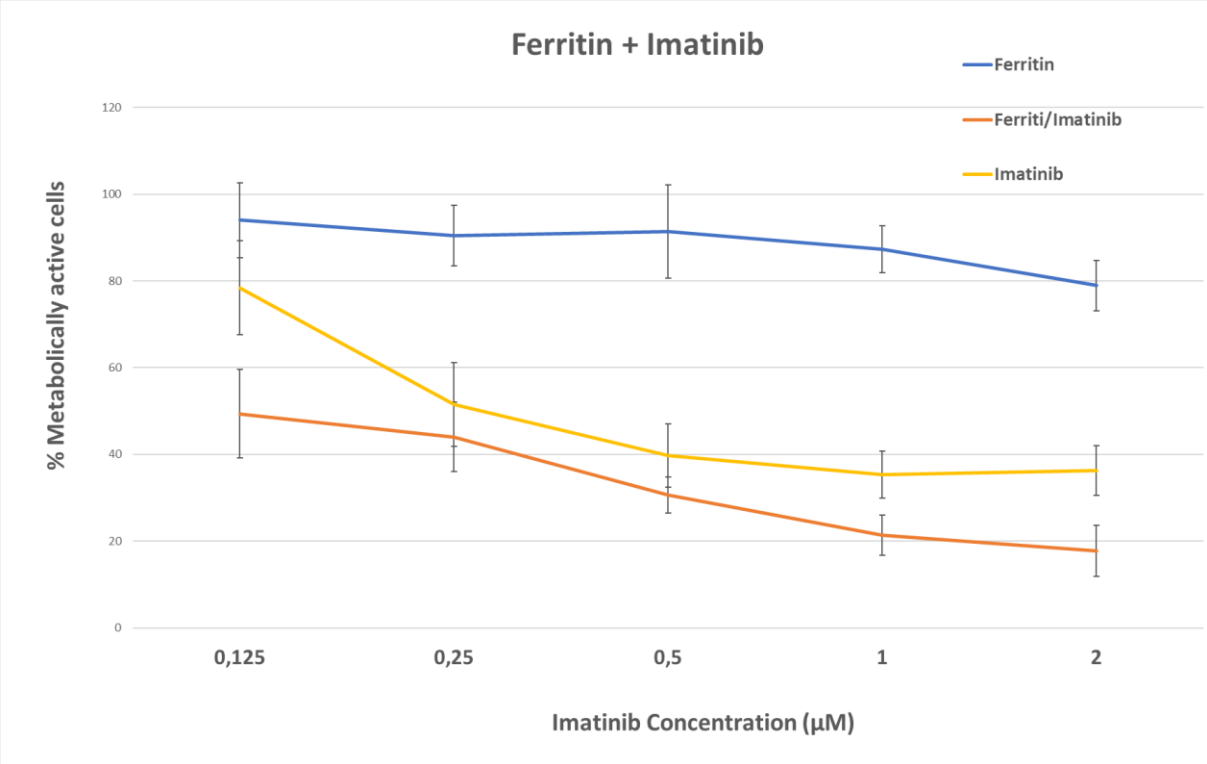


Figure 13 K562 metabolic effect study, performed under increasing imatinib concentrations and at a 1:100 ferritin: imatinib mol/mol ratio.

Table 2 IC_{50} of free Imatinib and Ferritin-Imatinib conjugation

Conditions	*IC_{50}*
Imatinib	0,245
Ferritin/Imatinib	0,125

3.2.2. Ferritin-Cisplatin based NMOF Metabolic Effects

Panc1 tumoral cells were incubated under two increasing cisplatin concentrations conditions. The first, ranging from 1.875 to 30 µM, figure S3, annexes, while the second ranging from 6.25 to 100 µM, figure 14. Ferritin and cisplatin were present at a 1:500 (figure S3) and at 1:100 (figure 14). Terbium was present in a 3:2 molecular ration with cisplatin.

At lower concentrations, from 1.875 to 30 µM, no differences between groups were observed.

At higher cisplatin concentrations, in figure 14, free cisplatin presented a higher metabolic effect, at 100µM was observed a 80% reduction effect on metabolic effect. However, no differences between the curves at lower concentrations was observed. Ferritin-cisplatin-terbium after urea treatment (FCT2) and without treatment (FCT1) did not present significant differences, thus being noticed any effect of the urea treatment.

Terbium, on other hand, showed a strong growth inducting potential, at high concentrations.

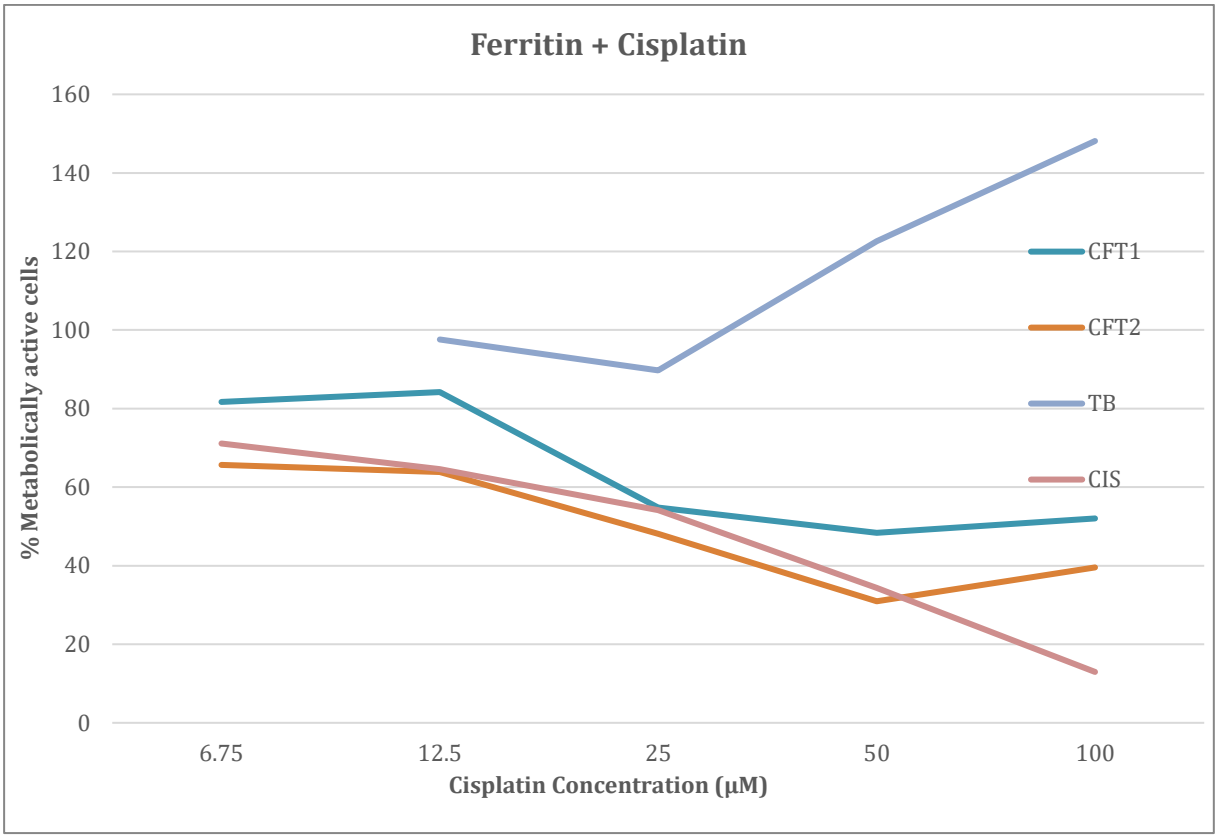


Figure 14 PANC1 NMOF conjugations metabolic effect study, performed under increasing cisplatin concentrations and at a 1:100 ferritin: cisplatin mol/mol ratio. Terbium:cisplatin Ratio was 3:2 mol/mol. CFT1- Ferritin/Cisplatin/Terbium formulation that did not pass through urea treatment; CFT2- Ferritin/Cisplatin/Terbium formulation that passed through urea treatment; Cis- free cisplatin; Tb-Terbium.

3.2.3. Ferritin-Cisplatin conjugations Metabolic Effects

Panc1 tumoral cells were incubated under increasing cisplatin concentrations, ranging from 6.25 to 100 µM. Cisplatin was incorporated into ferritin, in a 1:100 ferritin cisplatin ratio.

As it is possible to observe in figure S4, annexes, cisplatin-ferritin conjugates presented a decreased metabolism effect. NaCl and H_20 dissolved cisplatin did only present slight differences. When observing the plate, it was observed in the ferritin-cisplatin controls, several aggregates.

Cisplatin was then dissolved in an aqueous solution of apoferritin, in a 1:100 apoferritin cisplatin ratio. Figure 15 represents the metabolism assay curves, showing an increasing metabolism decreasing effect as the concentration increased. At higher concentrations, between 25 and 100 µM, no differences were observed between the samples, being the resulting metabolic activity very low at these concentrations. At lower concentrations, cisplatin incorporated by ferritin presented a metabolism decrease when compared to free cisplatin. The

IC_{50} of the latter was 6.35uM, while both free cisplatin presented higher IC_{50}, 12.5 and 14,5 µM respectively. Despite, only being observed slight differences between free cisplatin samples, 0.9% NaCl free cisplatin presented in general an increased metabolic effect. Free ferritin had a near 80% viability at all concentrations.

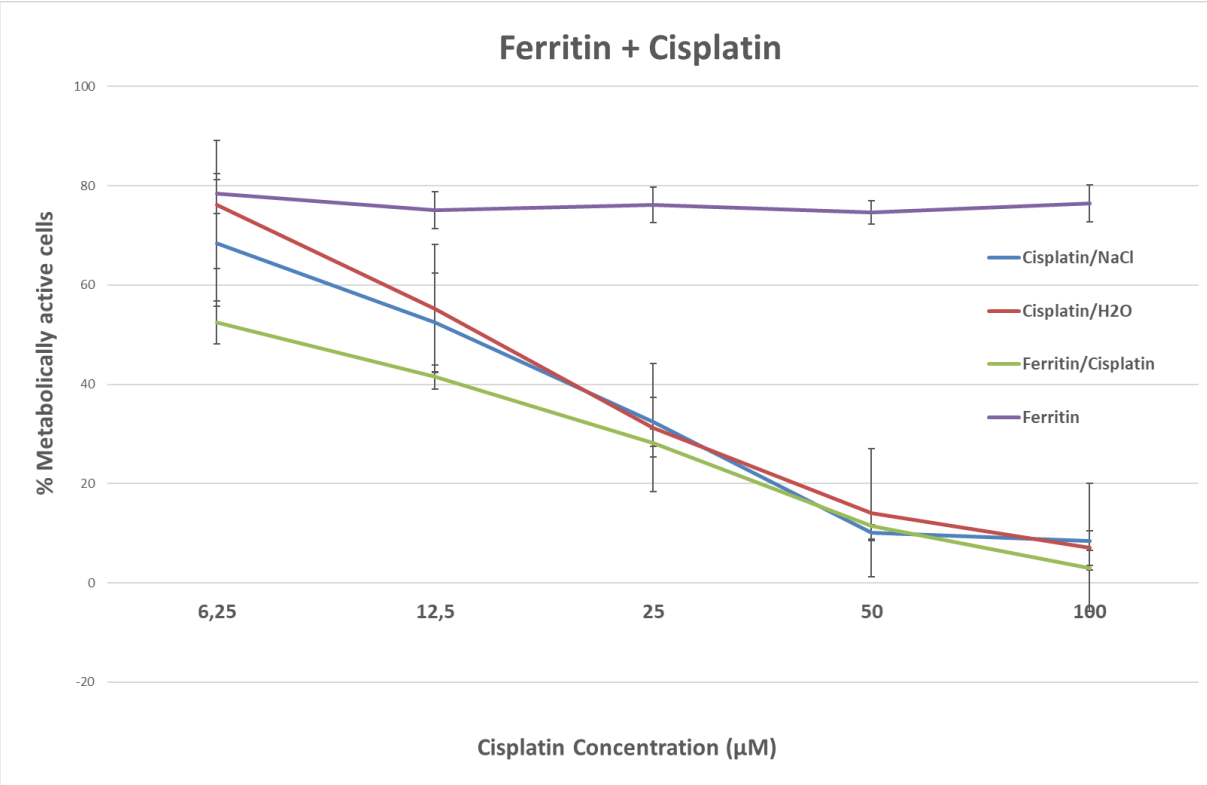


Figure 15 PANC1 Ferritin-Cisplatin conjugations metabolic effect study, performed under increasing cisplatin concentrations and at a 1:100 ferritin: cisplatin mol/mol ratio.

Table 3 IC50 of free Cisplatin (H_20 and 0,9% NaCl) and Ferritin-Cisplatin conjugation.

Conditions	***IC_{50}***
Cisplatin/H_2O	14,50
Cisplatin/NaCl	12,50
Ferritin/Cispaltin	6,25

3.2.4. TEM analysis on Panc1 cells treated with Ferritin-Cisplatin conjugation

Panc1 cells under no treatment, figure 16, were present and apparently normal. No chromatin was condensed at nucleic membrane periphery, with endoplasmic reticulum rough and smooth normal presence, a well-defined cellular membrane without pseudopod like structures presence.

However, ferritin-cisplatin compound presence, figure 17 and pseudopod like structures, mitophagy and vacuolization were observed. Furthermore, some endosome like structures were observed.

These structures were also found under free cisplatin presence, which also presented cytoplasmic matrix loss, figure 18A and 18B.

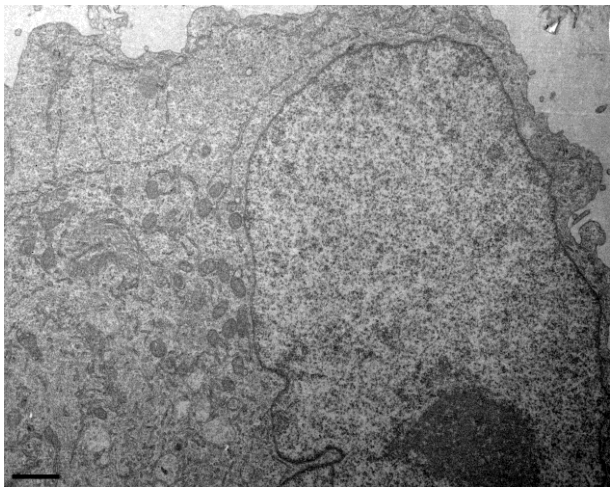


Figure 16 Panc1 TEM analysis. Organelles normality is observed. Extracellular Membrane is well defined (12000x).

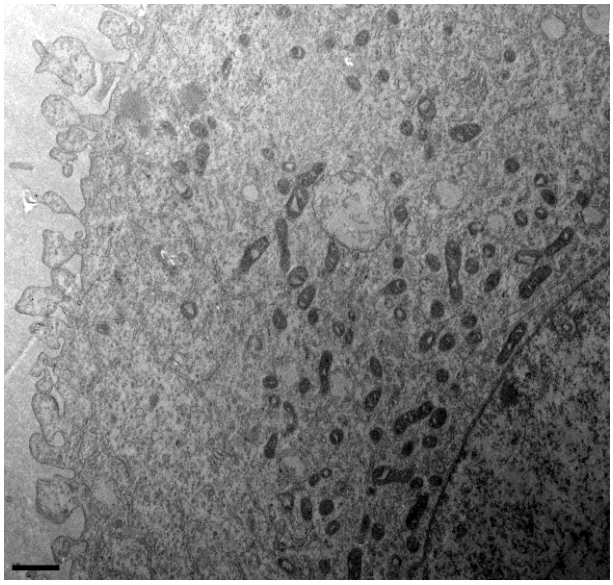


Figure 17 Panc1 treated with ferritin-cisplatin conjugation TEM analysis. Mitophagy, vacuolization and endosome like structures are observed. Extracellular Membrane pseudopods like structures (12000x).

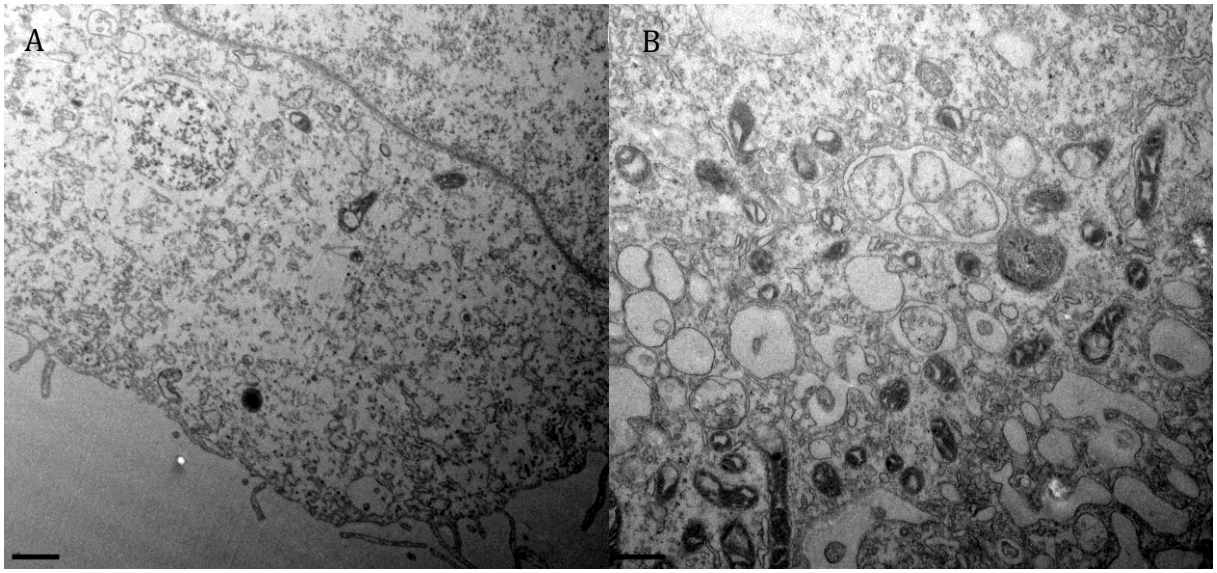


Figure 18 Panc1 treated with free cisplatin TEM analysis. Mitophagy, vacuolization and cytoplasmic loss are observed. Extracellular Membrane pseudopods like structures (A-12000x; B-25000x)

4. Discussion

Nanoscale particles have intriguing properties and potential applications, however from a synthetic point of view, the main challenge is to seek new procedures that allow the preparation of nanoparticles in a controlled manner, allowing a narrow size distribution, since its properties are tightly correlated with size and aggregation (Domínguez-Vera & Colacio, 2003). Preorganized biomolecular matrix provides a chemically and spatially confined environment for the homogeneous size nanomaterial synthesis, possibly being to the ideal vehicle.

The spherical 24 subunits ferritin shell is naturally able to incorporate iron into its apoferritin cage. This allows the generation of an intracellular ferric agglomerate composed of a maximum of 4500 iron atoms, as previously stated. Due to the size constrictions, since apoferritin hollow shell has an internal diameter of 8nm, this protein may only contain a fixed molecules number that may vary depending on the molecule type, namely its properties, like charge, molecular weight and size. However, to be able to be incorporated into the apoferritin shell, there is the need to firstly pass through apoferritin shell channels of 4A. Theoretically, no molecule bigger than the channel would be able to pass, however ferritin has the marvellous property of disassemble under high urea concentrations or at low *pH*. Circular dichroism (CD) analysis was performed, to observe the effect of these phenomena in the structure of ferritin. CD analysis is a widely used technique for the study of protein structure. Optically active macromolecules, such as proteins, exhibit differential absorption of circular polarized light, being the effect predominantly based on the excitation of electronic transitions in amide groups (Micsonai et al., 2015). Characteristic secondary structures such as α-helices, β-pleated sheets, turns, and disordered sections have specific Φ, Ψ dihedral angles and H-bond patterns affecting the CD spectrum.

The results, in general, showed an increased deformation as the acidification increases which correspond with the literature, observing the polishing of the ellipticity curve band, positive and negative as well, and even the slight disappearance of one negative band at pH 4. However, at pH 2 and 3 the expected polishing was not observed. Thus, we can infer that these conditions are possibly promoting only a slight ferritin denaturation and pore opening. However, under high concentration of Urea (8M) the curve is very irregular, demonstrating the conformation loss as expected.

Methanol initially did not promote protein denaturation. However, with the time passage a gradual denaturation of the ferritin, depending on concentration, was observed. As it was

pretended to simply observe methanol effects over time, with no specific concentration required, different timepoints were taken on different methanol concentrations. Denaturation is expected since methanol is a commonly used solvent by industries as antifreeze, fuel for small stoves, paint remover, windshield washer fluid and copying fluid. Poisoning with methanol can result in life-threatening metabolic acidosis, renal failure, blindness and even death (Jun, Xue, Liu, & Wang, 2011). Moreover, CD analysis of methanol effect on Haemoglobin also reported a secondary structure degeneration, with the loss of the a-helix typical conformation and the molecules probably adopting a looser conformation with extended polypeptide structures (Jun et al., 2011).

This ability to perform disassembly is followed up by ferritin ability to auto reassembly when removed from the denaturation condition in a nearly intact fashion. Transmission electron microscopy (TEM) analysis, allowed the confirmation that after methanol and urea depletion, the ferritin cage closes again which proves the conformation restauration, which is in conformity with the literature (Abe et al., 2016).

Thus, ferritin have a strong compound assimilation property that can be divided into two ways: pores diffusion entry and the disassembly followed by reassembly process. The disassembly-reassembly process is explored to incorporated compounds with a bigger size than the apoferritin cage pore size. Non-metallic compounds are usually incorporated via this method.

Due to this disassembly-reassembly effect, it is possible to encapsulate various compounds inside the cage. The compound release was tested on imatinib, after dissociation and reassembly process promoted by urea. Free imatinib is totally expelled from the dialysis solution after several hours, as is possible to observe in the control. In fact, in the various pHs conditions, no differences are found in the early stages of the experience, owing to the fact that free imatinib concentration is equal in every condition. After free imatinib removal phase, the pH variations lead to different release curves, being faster at lower pHs. The lower the pH is the more the protein loses its structure stability, and pores tend to open with the pH decrease (Domínguez-Vera & Colacio, 2003).

In the neoangiogenic process, a cancer hallmark, there is a strong angiogenesis promotion and vasculature growth, hastily done and lacking the normal consistence. Thus, the aberrant vessels are more permeable allowing a higher nanomaterials entry to the tumoral tissue. Coupled with the lack of lymphatic drainage, that to the residues non-removal, leads to an increase of the nanomaterials concentration at the place. This is called enhanced permeability and retention

(EPR) effect, by which molecules of certain sizes (like liposomes, nanoparticles, and macromolecular drugs) tend to accumulate in tumour tissue in a higher way than in normal tissues. So, ferritin may use this effect for the observed accumulation at the tumoral tissue (Kabat & Rohan, 2007), thus possibly leading to a higher apoferritin-drug complexes concentrations at the tumour. The acidic tumoral environment ensures a higher compound release at this local.

Moreover, the fact that cancer cells have an increased expression of ferritin receptors, such as transferrin receptor 1 (Tfr1) leads to a cancer targeting and a ferritin tumour cell endocytosis. Ferritin is internalized and may be lead to endosomes and lysosomes were the low pH further facilitates the compound release.

The EPR effect and ferritin increased internalization ensures a decrease of the possible therapy side effects, which coupled with the biocompatibility and non- immunogenicity of the protein by itself, leads to a great nanovehicle. However, cellular studies are needed to prove this hypothesis.

In order to test ferritin cage imatinib and cisplatin carrier potential in K562 and PANC1 cell lines, respectively, resazurin assay was performed. For that, cells, at least at 90% viability to ensure the proper cellular response, were cultivated in a 96 well plate for 24 hours, for cellular stabilization and adaptation to the new environment, prior to compound adding. K562 cancer cells were incubated under increasing imatinib concentrations. Panc1 cancer cells were incubated under increasing cisplatin concentrations. After 48 hours of incubation it was performed the metabolic evaluation by the resazurin assay. In order to avoid the direct toxic effects, cells were incubated for 3.5 hours after resazurin adding. In K562, it was firstly used the ferritin:imatinib 1:1250 ratio mol/mol, since the main goal was to brute force incorporation of imatinib. Thus, increasing the imatinib loaded quantity it was expected to improve the effect. However, this excess probably leads to a high free imatinib concentration. Therefore, no difference was found between free imatinib and imatinib loaded apoferritin (only one experiment was made under these conditions as preliminary test).

Nevertheless, a small difference between free imatinib and imatinib loaded apoferritin at lower concentration, was observed. Further, imatinib was loaded onto ferritin in a lower ratio, 1:100 mol/mol. In this condition, a higher effect was observed imatinib loaded apoferritin when compared to free imatinib. The IC50 obtained was 0.245µM for free imatinib and 0,125 µM for encapsulated imatinib. To note, that no ferritin toxicity was detected in this assay, as expected

since ferritin is a biocompatible protein already present in the tumour environment. Therefore, the increased metabolic effect observed by the encapsulated imatinib is not related with the ferritin alone but with the cellular uptake and direct imatinib release onto the cell driven by ferritin.

Regarding Panc1 cells, they were incubated with ferritin-NMOF (Ferritin-Cisplatin-Terbium compound). Ferritin/cisplatin (F/C) ratio was established at 1:500 mol/mol for a maximum incorporation. Terbium/cisplatin was 3:2 mol/mol. However, like imatinib, this low formulation F/C ratio showed no differences between free cisplatin and loaded cisplatin-terbium compound. Nevertheless, no effect was also observed in cisplatin free. For that reason, the initial ferritin-cisplatin-terbium concentration as well as the ratio between ferritin and cisplatin-terbium were increased. Although the free cisplatin at the highest concentration used (30µM) had a significant effect on the metabolic activity of the cells, the conjugations of cisplatin with ferritin and terbium did not show any benefit, and, in fact, was worse that free cisplatin. In fact, TEM analysis showed the presence of huge agglomerates in this condition. So, despite also being observed ferritin possibly loaded with cisplatin/terbium aggregates, the concentration to be used should be lower, thus explaining the obtained results. Furthermore, free terbium showed an increasing in the metabolic activity at the highest concentration.

Ferritin urea treatment promoted the disassembly of the cage. After, reassembly no differences were observed. Thus, the reassembly process does not affect drug efficacy or formulation toxicity.

Ferritin-cisplatin conjugation explored the cisplatin charge acquirement when in water solution. This may lead to a higher ferritin internalization, since it would be easier to pass through apoferritin pores. Firstly, no dialysis was performed, and ferritin was used. However, the effect was worse than free cisplatin.

After, analysing the solution, it was found that metal looking aggregates were formed. Due to this, it was proposed that iron and cisplatin may conjugate, forming large agglomerates. Thus, cisplatin was not being internalized into the cells, which lead to a lack of effect.

Therefore, iron was removed from ferritin, by dialysis performance. Here, the improvement effect on the cisplatin action was clear. At the highest concentration, free cisplatin was already extremely toxic, and no differences were found between cisplatin and cisplatin ferritin encapsulated. However, at lower concentrations, ferritin-cisplatin formulation had better effect

compared with free cisplatin, with an IC_{50} of 6,25 µM, contrasting with the 12,50 and 14,50 µM presented by free cisplatin in water and 0,9% NaCl, respectively.

To note, there was no differences between cisplatin dissolved in 0.9% of NaCl and dissolved in water. This might mean that despite its Cl^- loss, the cisplatin activity is not affected, which is not in accordance with literature (Schuldes, Bade, Knobloch, & Jonas, 1997).

TEM cellular images showed that after ferritin-cisplatin conjugation and free cisplatin treatment, Panc1 cells presented no viability signals. Like, pseudopods, with no well-defined cytoplasmic membrane. Furthermore, mitophagy and vacuolization were observed. Free cisplatin also presented cytoplasmic losses.

These results, showed that the metabolic effects decrease, measured recurring to resazurin, could be related with a cellular viability reduction.

The ICP-MS analysis showed that cisplatin in water was incorporated at lower amounts, which is not in accordance with the hypothesis tested in this work. However, the dialysis solution was the double than in the beginning of dialysis, possibly due to a bad isolation. TEM analysis showed small electrodense compounds which appeared to be ferritin incorporated. Further tests are required in order to validate the improvement or not of the encapsulation efficiency by water positive charge promotion.

5. Conclusions and Future Perspectives

In this work ferritin was shown to be an interesting drug delivery vehicle, regardless the internalization method, disassembly/reassembly or direct apoferritin pores diffusion. Ferritin was proven stable, even after the disassembly-reassembly, not being observed any side effects against the cells due to this process.

Imatinib internalized into ferritin was shown, for the first time, to be more effective than free imatinib against the K562 cell line, with an IC50 of 0,125µM contrasting with the 0,245 µM of the free imatinib.

Cisplatin was internalized by promoting an initial cisplatin positive charge, that should lead to a higher encapsulation efficiency. Thus, reflecting in a higher metabolic effect on Panc1 cell line, 6,25 µM, when compared with free cisplatin (12,50 and 14,50 µM, for 0,9% NaCl and for the water dissolved cisplatin, respectively). However, NMOF formulations did not present good results, and further optimizations will be required.

Unfortunately, ICP-MS analysis showed a lower encapsulation efficacy of water dissolved cisplatin. However, cellular effects and TEM analysis are reflecting the opposite. Thus, new analysis are required.

Thus, further studies are required, to provide more information about the ferritin potential as a drug delivery system. Imatinib encapsulation studies, proved the possibly of incorporating a whole batch of compounds and not only metallic compounds. Therefore, highly efficient drugs against cancers but not possible to provide to patients, due to their properties may now gain a new opportunity. It is required to research ferritin targeting capacity, to access to possible treatment side effects reduction brought by this protein.

To conclude, these studies need to be carried on, using other cell lines and animal models, aiming to confirm the enhanced anticancer activity and verify the mechanism of action of these conjugations, as well the ferritin encapsulation properties.

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Annex

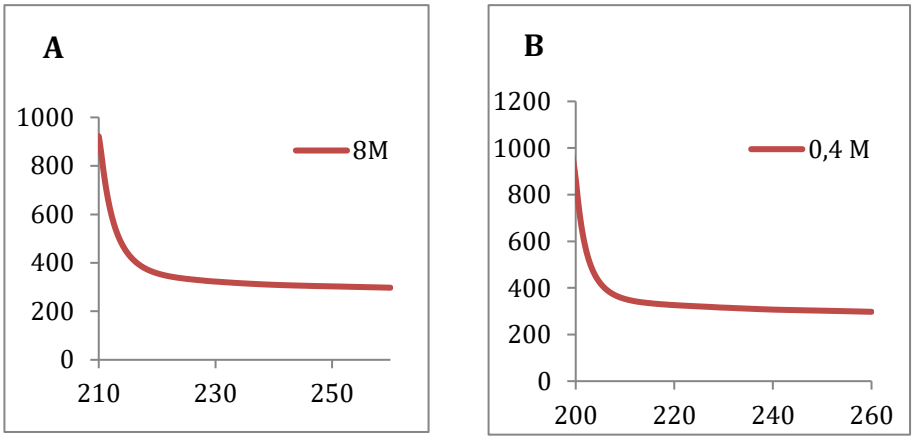

Figure S 1 HT values under urea treatment. A) Urea 8M; B) Urea 0,4M; The graphs started in 260 nm and went till HT reached around 800V.

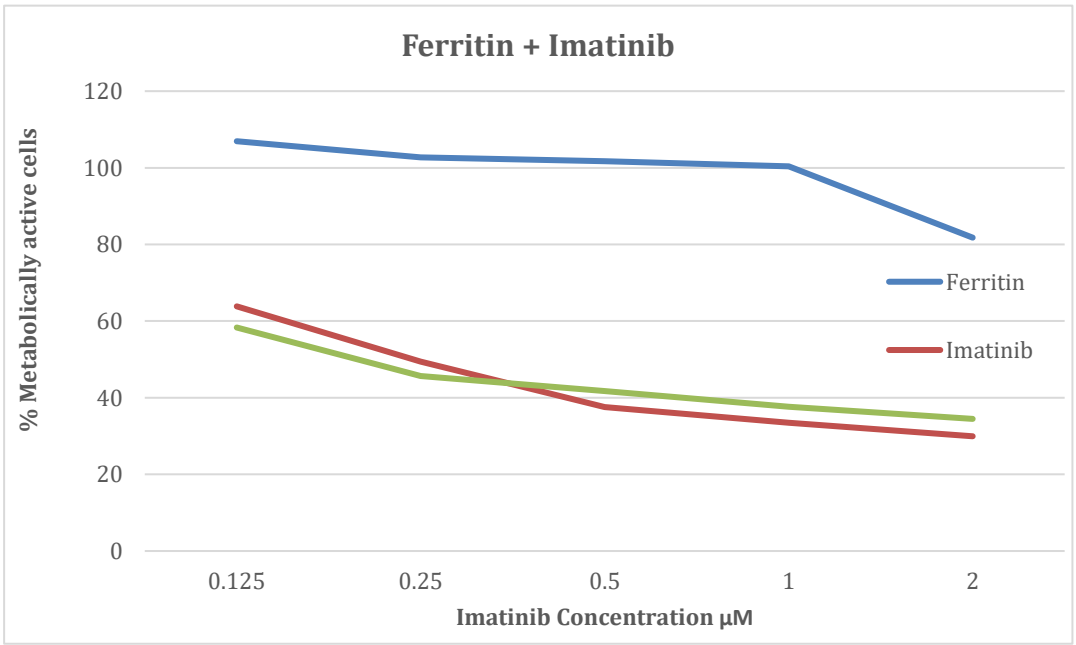

Figure S 2K562 metabolic effect study, performed under increasing imatinib concentrations and at a 1:1250 ferritin: imatinib mol/mol ratio.

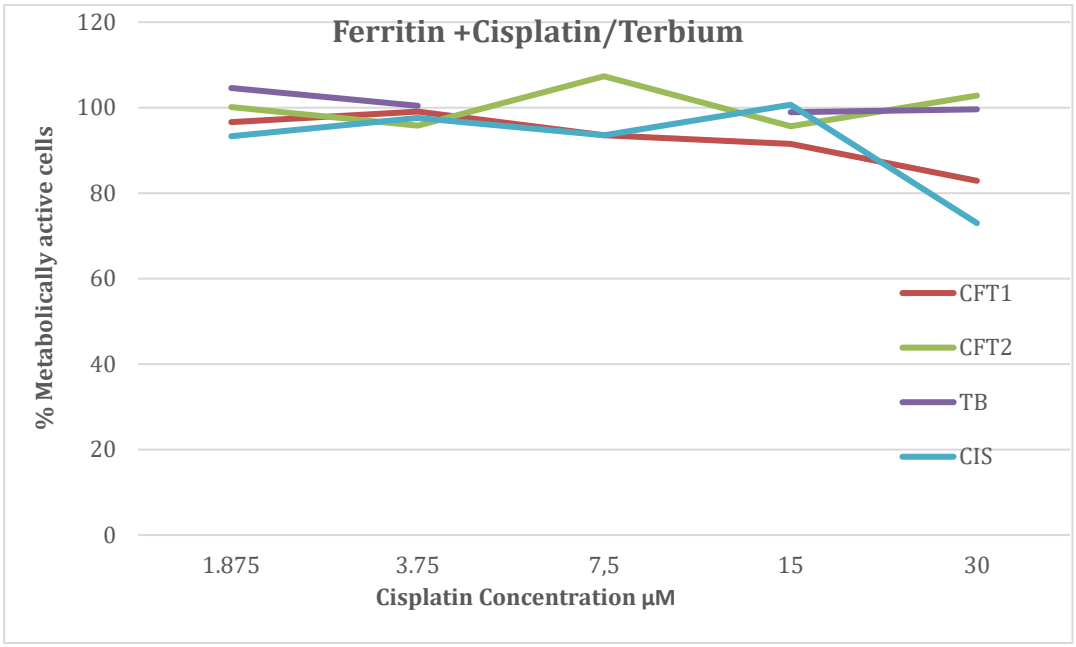

Figure S 3 PANC1 Ferritin-NMOF conjugations metabolic effect study, performed under increasing cisplatin concentrations and at a 1:500 ferritin: cisplatin mol/mol ratio. Terbium:cisplatin Ratio was 3:2 mol/mol. CFT1- Ferritin/Cisplatin/Terbium formulation that did not pass through urea treatment; CFT2- Ferritin/Cisplatin/Terbium formulation that passed through urea treatment; Cis- free cisplatin; Tb-Terbium.

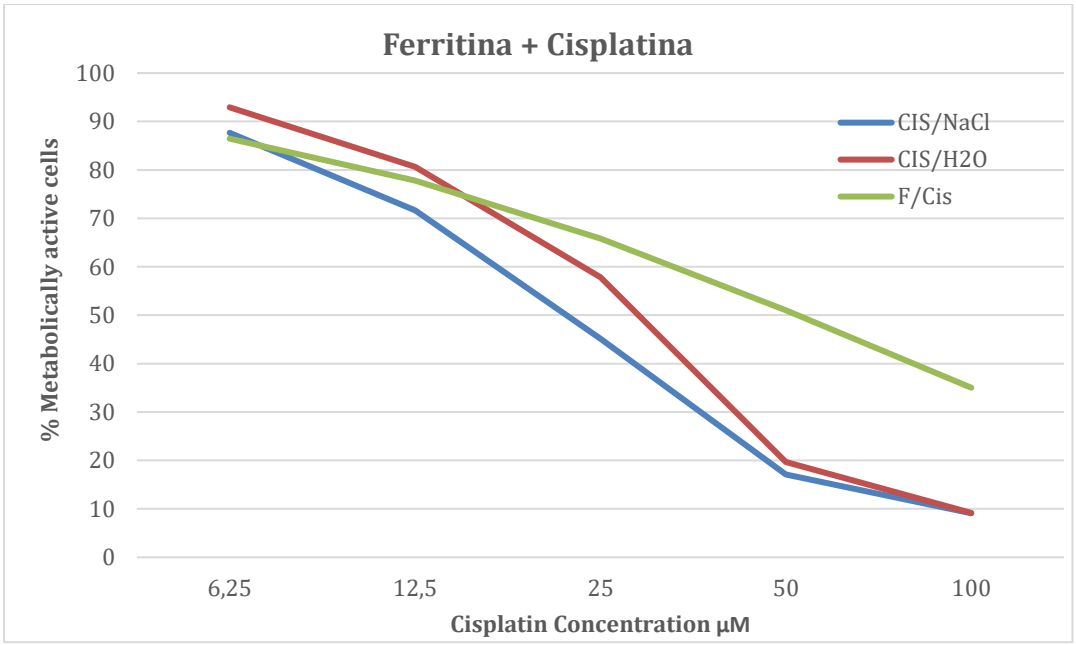

Figure S 4 PANC1 Ferritin-Cisplatin conjugations metabolic effect study, performed under increasing cisplatin concentrations and at a 1:100 ferritin: cisplatin mol/mol ratio.