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Supervisor
Ch. Prof. Flavio Rizzolio

Graduand

Kossivi Jean-d'arc
Pacome Andele

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ABSTRACT

Cancer is a complex disease that can start mainly in any organ in the body in which abnormal cells grow in an uncontrolled manner to invade neighboring parts of the body to spread to other organs. This disease is the second leading cause of death worldwide, accounting for approximately 9.6 million deaths, or one in six deaths, in 2018. The incidence is continuously increasing due to population's aging, cancer-causing behaviors and pollution[1]. Cancer is associated with genetic diversity and other factors that also can create phenotypic diversity within a cancer cell population. These factors include DNA and histone modifications, transcriptional, post-transcriptional regulations and protein aberrant activities. In cancer therapy, proteins that control multiple oncogenic pathways are among the best targets.

One protein involved in multiple cellular pathways is the peptidylprolyl PIN1, an isomerase formed by a WW domain and a catalytic PPase domain with a molecular weight of 18 KDa. The isomerase domain of PIN1 mediates the rapid cis or trans conformation of the prolyl bond and speed up this reaction to 1000-fold. This modification can result in a conformational change of the target protein causing alterations in its function, stability and intracellular localization. PIN1 regulates proteins involved in cell-cycle progression and can operate as a molecular timer of this important process.

The project aimed to design a drug delivery system for a PIN1 inhibitor (C17) that was published by our group in 2019 [8]**.** For this purpose, C17 was solubilized with the FDA approved surfactant pluronic acid F127 (F127) and coated with albumin (F127-albumin) as drug delivery system.

To evaluate these biomaterials, C17, C17-F127 and C17-F127 albumin were analyzed using scanning electron microscopy (SEM), Transmission electron microscopy (TEM) and Dynamic light scattering (DLS). Through these experiments, it was possible to characterize the morphology of the modified compounds and to measure the size of the nanoparticles, the coating, the zeta potential and poly-dispersion index (PDI). Finally, cell viability was performed to assess the effectiveness of the inhibitory substance. IC50 values comparison between the free form and drug encapsulated with F127 and F127-albumin revealed that the novel drug delivery systems are more effective on killing cancer cells than free C17 compound.

1. Introduction:

1.1. THE CONCEPT OF CANCER

1.1.1 EPIDEMIOLOGY

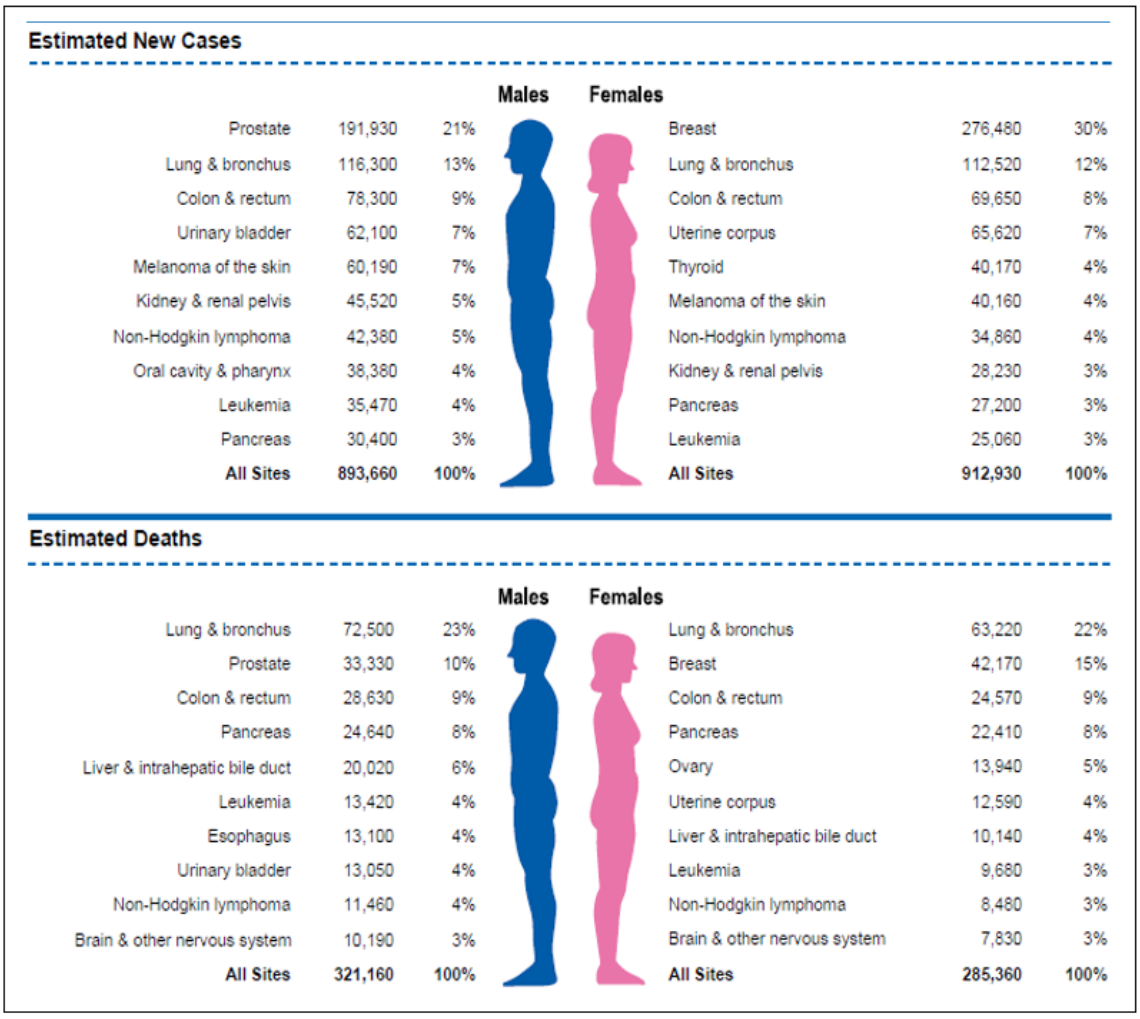
Cancer is a complex disease that could initiate in any organ of the body when abnormal cells grow in an uncontrolled manner to invade neighboring parts of the body and spread to other organs. Cancer is a multifactorial disease caused by a group of genetic, behavioral, and environmental factors. It is characterized by alterations in cell physiology including self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastatic potential, limitless proliferation, sustained angiogenesis and evasion of cell death. This disease is the second leading cause of death worldwide, accounting for approximately 9.6 million deaths, or one in six deaths, in 2018.

A steadily increasing proportion of elderly people in the world will result in approximately 16 million new cases of cancer by the year 2020 according to International Agency for Research on Cancer (IARC).

According to the World Health Organization (WHO), approximately 70% of deaths from cancer occur in low- and middle-income countries. The associated incidence is continuously increasing as the population is growing and aging and as the cancer-causing behaviors and pollution are increasing [1].

The five most common cancer types among women include cancers of the breast, lung, colon and rectum, uterine, and thyroid. Among men, the most common cancer sites include prostate, lung, colon and rectum, urinary bladder, and melanoma of the skin [9]. While there is a considerable overlap in common types of cancers worldwide, developing countries tend to have a higher incidence and death rates for viral infection–related cancers such as hepatitis-related liver cancer and human papillomavirus (HPV)-related cervical cancer.

In **figure 1** are depicted the most common cancers expected to be diagnosed in men and women in 2020. Prostate, lung and colorectal cancers (CRCs) account for 43% of all cases in men, with prostate cancer alone accounting for more than 1 in 5 new diagnoses. For women, the most common cancers are breast, lung, and colorectal, accounting for 50% of all new diagnoses [10].



Estimated New Cases

Males				Females		
Prostate	191,930	21%		Breast	276,480	30%
Lung & bronchus	116,300	13%		Lung & bronchus	112,520	12%
Colon & rectum	78,300	9%		Colon & rectum	69,650	8%
Urinary bladder	62,100	7%		Uterine corpus	65,620	7%
Melanoma of the skin	60,190	7%		Thyroid	40,170	4%
Kidney & renal pelvis	45,520	5%		Melanoma of the skin	40,160	4%
Non-Hodgkin lymphoma	42,380	5%		Non-Hodgkin lymphoma	34,860	4%
Oral cavity & pharynx	38,380	4%		Kidney & renal pelvis	28,230	3%
Leukemia	35,470	4%		Pancreas	27,200	3%
Pancreas	30,400	3%		Leukemia	25,060	3%
All Sites	**893,660**	**100%**		**All Sites**	**912,930**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	72,500	23%		Lung & bronchus	63,220	22%
Prostate	33,330	10%		Breast	42,170	15%
Colon & rectum	28,630	9%		Colon & rectum	24,570	9%
Pancreas	24,640	8%		Pancreas	22,410	8%
Liver & intrahepatic bile duct	20,020	6%		Ovary	13,940	5%
Leukemia	13,420	4%		Uterine corpus	12,590	4%
Esophagus	13,100	4%		Liver & intrahepatic bile duct	10,140	4%
Urinary bladder	13,050	4%		Leukemia	9,680	3%
Non-Hodgkin lymphoma	11,460	4%		Non-Hodgkin lymphoma	8,480	3%
Brain & other nervous system	10,190	3%		Brain & other nervous system	7,830	3%
All Sites	**321,160**	**100%**		**All Sites**	**285,360**	**100%**

Figure 1: Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex, United States, 2020 [10] . Estimates are rounded to the nearest 10 and exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder. Ranking is based on modeled projections and may differ from the most recent observed data.

1.1.2 THE BENIGN AND MALIGN CONCEPT

A tumor is a pathologic disturbance of cell growth, characterized by excessive and abnormal proliferation of cells. Tumors consist of an abnormal mass of tissue which may be either solid or fluid filled. Benign tumors consist of non-cancerous cells that stay localized in one place. To become cancerous, normal cells must develop mutations that allow them to no longer obey the

boundaries of adjacent cells, thus allowing them to grow uncontrollably and enabling them to produce their own blood supply. Malignant tumors are made of cancer cells that also gain the ability to invade the basement membrane and surrounding tissue, enter the bloodstream, and in most cases, metastasize to distant organs.

A benign tumor is less harmful unless it does not confine with any important organs, tissues, nerves, or blood vessels, causing damage. Fibroids in the uterus and breast, polyps of colon and moles are some examples of benign tumors. Benign tumors can be removed by surgery. They can grow very large, sometimes weighting pounds. They can be dangerous, such as when they occur in the brain and crowd the normal structures in the enclosed space of the skull. Also, some types of benign tumors such as intestinal polyps are considered as precancerous and are removed immediately to impede them becoming malignant thus preventing colon cancer. They don't reoccur when removed.

Rarely some types of benign tumors transform into malignant tumors. But tumors like adenomatous polyps (adenomas) in the colon have a greater risk of transforming into malignant tumor.

Malignant means that the tumor is made of cancer cells and it can invade nearby tissues. Some cancer cells can move into the bloodstream or lymph nodes, where they can spread to other tissues within the body; breast cancer begins in the breast tissue and may spread to lymph nodes in the armpit if it's not detected and treated at an early stage. Once breast cancer has spread to the lymph nodes, the cancer cells can travel to other areas of the body, like the bone or liver. The breast cancer cells can then form tumors in those locations referred as secondary tumors. A biopsy of these tumors might show characteristics of the original breast cancer tumor.

Some specific differences between the two types of tumor behavior are: the malignant tumors grow more rapidly than benign tumors, they use to invade the tissues around them by the penetration of the basal membrane that surrounds normal tissues and spread to other parts of the body using the bloodstream or the lymphatic system.

At the microscope, cancer cells often have abnormal chromosomes and DNA, making their nuclei larger and darker. They also often have different shapes than normal cells (**Figure 2**).

There are some benign tumors that secrete hormones, such as benign pheochromocytomas, malignant tumors are more likely to do so. Malignant tumors can secrete substances that cause

effects throughout the body, such as fatigue and weight loss. This is known as paraneoplastic syndrome [11].

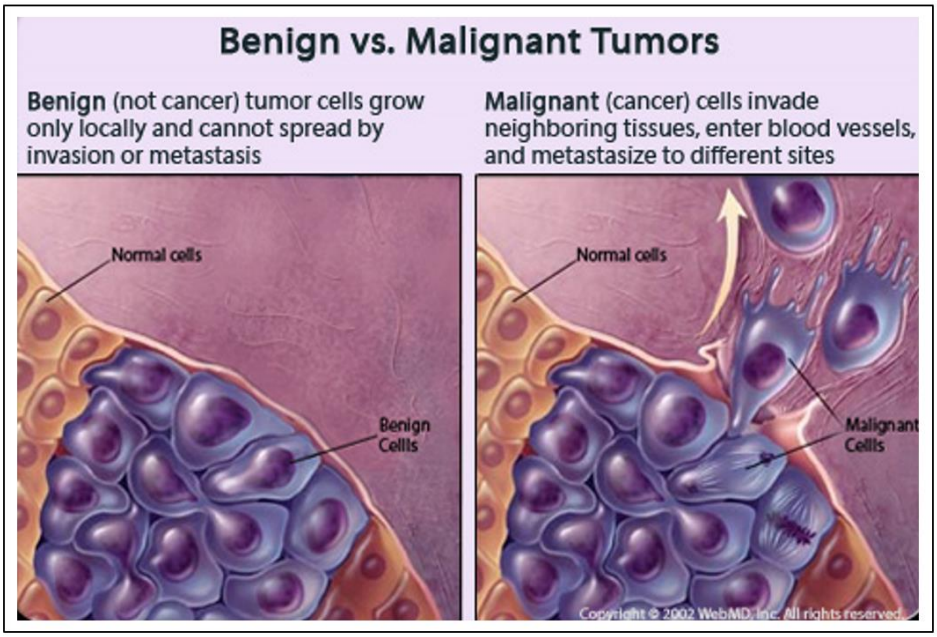


Figure 2: Benign and malignant tumor cells. The morphological differences between benign cells and malignant cells. (From: MedicineNet.com)

1.1.3 CLASSIFICATION OF CANCER

The basic classification of cancer relies on the type of tissue in which the tumor originates (histologically) and on the primary site or location in the body where the cancer first developed. According to the international standard for the classification and nomenclature of histology, the International Classification of Disease for Oncology, third Edition (ICD-O-3), cancers are grouped into six major categories:

- Carcinoma: refers to a malignant tumor of epithelial origin or of the internal or external lining of the body. Carcinomas account for 80 to 90 percent of all cancer cases. Epithelial tissue is present in the skin, as well as the covering and lining of organs and internal passageways, such as the gastrointestinal tract.

  Carcinomas are divided into two major subtypes:

  - Adenocarcinomas which develops in a gland or an organ, generally occur

in mucus membranes and are first seen as a thickened plaque-like white mucosa. They spread easily through the soft tissue where they start.
-Squamous cell carcinoma which originates in the squamous epithelium, they occur in many areas of the body.
Most carcinomas affect organs or glands responsible of secretion, such as the breast, the lungs, the colon, the prostate or the bladder.

- Sarcoma: refers to neoplasms that originates in the connective and supportive tissues including bones, tendons, cartilage, muscles and fat. Generally, it occurs in young adults, commonly developing a painful mass on the bones. Sarcoma tumors usually resemble the tissue in which they grow.

Some examples of sarcomas include:

- Osteosarcoma or osteogenic sarcoma affecting the bones
- Chondrosarcoma affecting the cartilage
- Leiomyosarcoma affecting the smooth muscle
- Rhabdomyosarcoma affecting the skeletal muscle
- Mesothelial sarcoma or mesothelioma affecting the membranous lining of body cavities
- Fibrosarcoma affecting the fibrous tissue
- Angiosarcoma or hemangioendothelioma affecting the blood vessels
- Liposarcoma affecting the adipose tissue
- Glioma or astrocytoma affecting the neurogenic connective tissue found in the brain
- Myxosarcoma affecting the primitive embryonic connective tissue
- Mesenchymous or mixed mesodermal tumor affecting the mixed connective tissue types

- Myeloma: refers to the cancer that originates in the plasma cells of bone marrow. The plasma cells are also called plasma B cells, the white blood cells secrete large quantities of proteins called antibodies in response to the presented specific substances called antigens.

- Leukemia: refers to the liquid cancers or blood cancers, also cancers of the bone marrow. It is often associated with the overproduction of immature white blood cells, unable to perform the normal function, therefore the patient is often more susceptible to infection. Leukemia also affects the red blood cells inducing anemia, causing poor blood clotting and fatigue.

Some examples of leukemia include:

- Lymphatic, lymphocytic, or lymphoblastic leukemia affecting the lymphoid and lymphocytic blood cell series
- Polycythemia vera affecting various blood cell products, but with red cells predominating
- Myelogenous or granulocytic leukemia affecting the myeloid and granulocytic white blood cell series.

- Lymphoma: refers to the cancer in the glands or nodes of the lymphatic system consisting of a network of nodes, vessels, and organs such as the spleen, tonsils, and thymus responsible for the purification of bodily fluids and production of lymphocytes. Lymphomas are solid cancers that may occur in specific organs such as the stomach, breast or brain.

They are subclassified into two categories:

- Hodgkin lymphoma: typically begins in the upper body, such as the neck, chest or armpits.
- Non-Hodgkin lymphoma: may arise in lymph nodes anywhere in the body.

In Hodgkin lymphoma, the presence of Reed-Sternberg cells, abnormal lymphocytes that may contain more than one nucleus, diagnostically distinguishes Hodgkin lymphoma from Non-Hodgkin lymphoma.

- Mixed Types: refer to the type components that groups different categories together. Some examples are:

- Adeno-squamous carcinoma
- Mixed mesodermal tumor, carcinosarcoma and teratocarcinoma.

From: https://www.cancer.gov/

1.1.4 CAUSES OF CANCER

Cancer can be caused by a range of factors. Although cancers are considered as hereditary disease, most cases are in fact, sporadic in nature. Other important factors to consider when discussing the causes of cancer may include:

- **Exposure to chemical and physical agents**:

This category comprises various chemicals that have potent causal effect on many types of cancers. Over 90% of lung cancer cases have been attributed to the chemical components of tobacco smoke, and to a broader extent, they may also cause cancers of the head, neck, or esophagus. Alcohol is another chemical that humans are commonly exposed to and reports have stated that it is one of the primary chemical agents which may trigger liver cancer. In terms of physical agents, asbestos exposure can be regarded as one of the most traditional examples of how individuals may suffer from mesothelioma

- **Obesity and diet**:

Studies have shown a strong correlation between the obesity status of individuals and the risk of contracting cancer in their lifetime. Examples of how diet may cause cancers include betel nut which may trigger oral cancer and a diet high in salt which may cause gastric cancer. The effect of weight is strongest for post-menopausal breast cancer and cancers of the gall bladder, kidney and endometrium. Approximately 5% of all incident cancers in Europe could be prevented by maintaining one's BMI (body-mass index) and without exceeding dietary supplements such as vitamins or other micronutrients. For example, the consumption of foods containing beta-carotene was reported to be correlated with a reduced risk of lung cancer. Various cancers were likely to be reduced by uptake of food containing proper amounts of carotenoids, vitamins C and E and selenium. Furthermore, Aspirin and folate supplements were shown to reduce colorectal cancer incidence.

- **Hormones**:

Reproductive factors effect on breast and ovarian cancer have been assumed to reflect underlying hormonal processes, which is confirmed by the effects of both endogenous and exogenous hormones. Breast cancer incidence increased after pregnancy and reduced with estrogens administration as oral contraceptives or hormone replacement therapy. In contrast, it is permanently lowered by other factors such as late menarche, early menopause, early first child delivery and a high number of pregnancies. Hormone replacement therapy was also associated with endometrial cancer.

- **Infection**:

A chronic gastric bacterial infection by *Helicobacter pylori* can cause gastric ulcers, a major factor in the development of stomach cancer. In addition, more than 100 human papillomaviruses (HPVs) have been sequenced, and DNA from a phylogenetic subgroup of sexually transmitted HPVs, are detectable in virtually all cervical cancers worldwide. These and other HPVs may also cause cancers of other sites (head and neck, esophagus and skin). The hepatitis-B virus (HBV) contributes to liver cancer in high-incidence regions. The hepatitis-C virus (HCV) is carcinogenic as well.
Other pathogens that cause cancer risk include:

- Epstein–Barr virus (EBV): associated with various B-cell malignancies and nasopharyngeal cancer.
- Malaria (the major cofactor with EBV): associated with Burkitt's lymphoma in Africa
- Human T-cell lymphotropic virus type: associated with some T-cell leukemias and lymphomas
- HIV: associated with non-Hodgkin's lymphoma
- Human herpes virus: associated with Kaposi's sarcoma
- Schistosomiasis: associated with bladder and colon cancer
- Liver flukes: associated with cholangiosarcoma

More minor causes of cancer may include prolonged exposure to ionizing radiation, autoimmunity, and lifestyle, all of which would contribute to the epigenetic modulation which may give rise to cancers [12].

1.2 MOLECULAR PATHWAYS IN CANCER

The onset of cancerous conditions is due to an accumulation of multiple genetic mutations leading to the deregulation of signaling pathways that control cell growth, apoptosis, and DNA repair [13]. Once activated, these pathways induce cellular controls impairment, thus cancer cells can proliferate and grow in the absence of normal restrictions. The well-defined means by which normal cells transform into cancerous ones consist of a number of acquired molecular, biochemical, and cellular features that result from alteration of key pathways. **Figure 3** depicts the progression of cancer from a single cell to metastatic tumor.

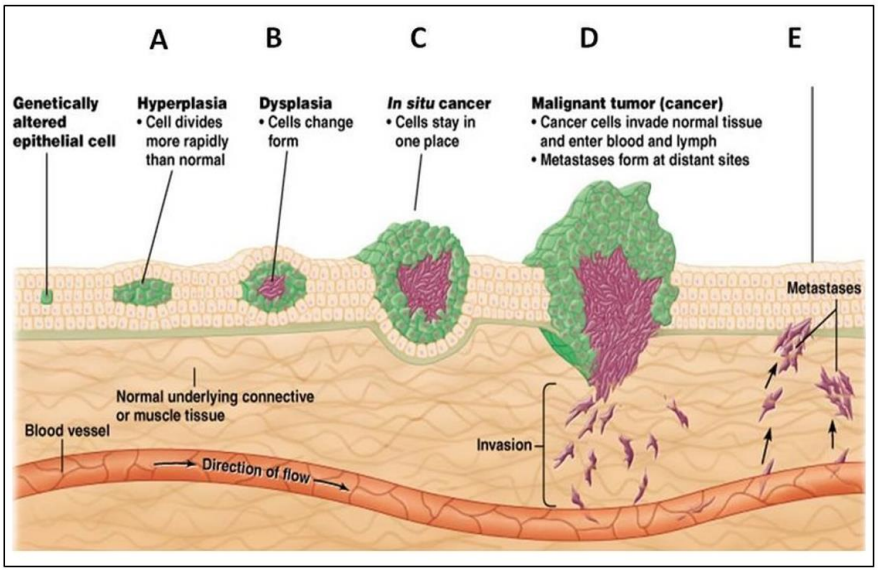


Figure 3: Stages of tumor development and mechanism of metastasis [14].

1.2 .1 SOMATIC MUTATIONS AND CANCER

Two specific gene classes play a key role in carcinogenesis: oncogenes and tumor suppressor genes (**Figure 4**). The accumulation of activated oncogenes and the inactivation of tumor suppressor genes subsequently confer the abnormal behavior that characterize cancerous cells. Proto-oncogenes, the precursors to oncogenes, are altered by dominant mutations, conferring a gain of function increasing the proliferation to a normal cell. These genes enhancing the proliferative capability of cells are termed oncogenes in their mutated form. On the other hand, tumor-suppressor genes, are inhibited [15]. The result of mutations in both classes of genes affects fundamental cellular processes including metabolism, growth, proliferation, and death. Consequently, the cancerous cells gain the ability to grow in number, forming tumors at the local site.

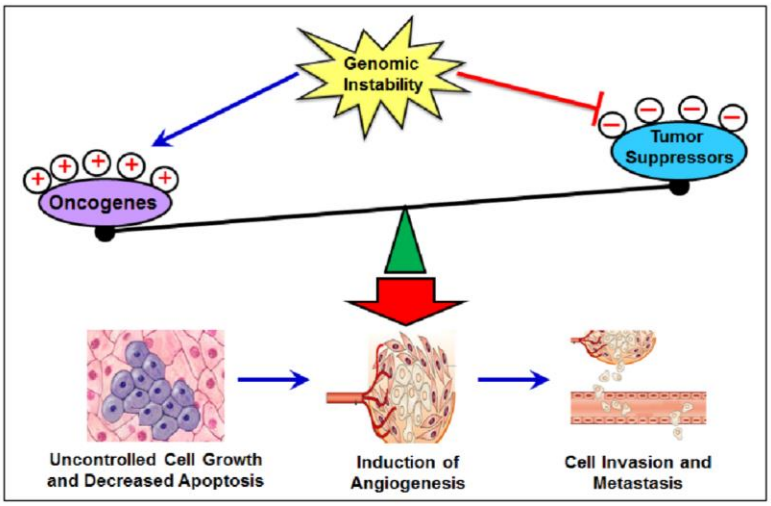


FIGURE 4: Oncogenes, tumor suppressor, and cancer [16]. Genomic instability caused by various factors such as viruses, cytotoxic drugs, and ionizing radiation triggers mutations in oncogenes or tumor suppressor genes and perpetuates the unstable genome on the way to malignancy

1.2.1.1 ONCOGENES

Dominant mutations in the normal form of the gene, the proto-oncogene, leads to the activation of oncogenes. This activation can be induced by the alteration of a single allele responsible of normal cellular function. Consequently, oncogene activation, may induce cancerous cell

formation since these genes are involved in tumor formation and maintenance by activating a certain metabolic pathway for cellular growth.

Various oncogenes act by inhibiting apoptotic pathways, or by negatively inhibiting genes that control cellular differentiation. They alter specific mechanisms by using signals for normal growth, to stimulate proliferation [17].

In addition, oncogenic activation is essential for cellular immortalization such as in HPV-caused cancers [18] and is also involved in altering cellular metabolism to support proliferation such as altered glucose and glutamine metabolism in tumors [19].

Particularly, a proto-oncogene called c-Myc regulates multiple metabolic pathways important for growth in non-cancerous cells and cancerous cells. In non-cancerous cells, the transcription factor Myc regulates many processes that control the entry to the cell cycle, cellular growth and proliferation [20]. In normal conditions, growth factors stimulate Myc activation. However, in its mutated form, the oncogenic c-Myc is overexpressed, thereby increasing the rates of glycolysis and the expression of enzymes functioning in nucleotide and amino acid metabolism, thus altering specialized biosynthetic activities to favor cell division and cancer growth.

Another mutation in Ras genes have been found in over 20% of human cancers [21]. The Ras gene family encodes for proteins crucial to transmit signals from receptors to downstream regulators of survival and growth. These genes play key role in cancer development, as the mutated forms of Ras genes means more Ras signaling proteins [22].

K-Ras is the most mutated Ras gene encoding for Ras protein signals in cancer. K-Ras generally functions in stabilizing Myc, that both in their mutated forms can cooperate to stimulate tumorigenesis.

1.2.1.2 TUMOR SUPPRESSOR GENES

Like oncogenes, mutations in tumor suppressor genes (TSG) also induce the deregulation of cellular signaling pathways, consequently leading to changes in gene expression [22]. The normal function of tumor suppressor genes is to inhibit the attributes that characterize cancer growth. Some tumor suppressor genes encode for proteins that directly regulate the integrity of the genetic material by repairing DNA damage while others function in regulating the extracellular microenvironment [23]. Generally, TSGs function in monitoring cellular growth, apoptosis,

replication, metastasis and angiogenesis. Mutations of these genes favor the classical conditions that characterize cancer. Unlike oncogenes, the altered TSGs become inactive and confer a loss of function only if both alleles are mutated; mutation of the first allele serves as a predisposition to tumor development, whereas the second allele mutation results in tumor initiation [24]. Nevertheless, a simple epigenetic modification of a single allele of a TSG may be enough to induce tumorigenesis if the mutated allele gets deleted [25].

A well-known TSG to be altered in cancer formations is the p53 tumor suppressor gene coding for the nuclear p53 protein. Approximately 50% of human tumors contain cells that have defective checkpoint pathways due to mutated p53 and is most common in ovarian cancer. P53 is a transcription factor that prevent genetic instability by regulating transcription of several genes involved in the cell cycle [26]. The activation of p53 via post-translational modifications is a response to several stimuli, such as DNA damage, hypoxia, and oxidative stress [27]. Cells respond to the inducing expression of p53 by arresting the cell cycle, senescence, differentiation, or by activating the apoptotic cascade in case of excessive damage.

The TSG encoding for p53 is often altered by a point mutation in one allele, and chromosomal deletion of the second allele, leading to genetic alterations in the DNA-binding site of p53. It has also been shown that inactivating conformational changes can inhibit normally functioning p53 protein by altering its DNA-binding domain [28].

1.2.1.3 EPIGENETIC MODIFICATIONS IN CANCER

The term, “epigenetics” refer to the complex interactions between the genome and the environment that are involved in development and differentiation in higher organisms.

The epigenetic regulations of a cell are determined by DNA methylation, histones covalent modifications, and non-coding RNAs and networking with each other. This regulatory system is important in some diseases that are caused by defects in the epigenetic system including cancer. Most of the cells in the body have the same genome, however the epigenetic system of the cells regulates the different features and function.

Various mechanisms are responsible for changing the expression of a gene epigenetically:

- DNA methylation is commonly used to silence genes expression in eukaryotic cells, it is accomplished by adding a methyl group to C5 of the cytosine ring by DNA. DNA

methylation condenses the chromatin resulting in transcriptional inhibition. The importance of DNA methylation in cellular biology has been identified, including embryonic development, chromosome X inactivation and gene expression timing at different stages of evolution.

In cancer, the aberrant methylation affects TSG genes, such as P53 and P16 inhibiting their expression and it is involved in drug resistance as well. Hypermethylation can also be used as an early diagnosis biomarker such in colon cancer [29].

- Histone modifications include phosphorylation, methylation and acetylation:
  - Phosphorylation: the addition of phosphate increases the negativity of the histones, affecting the chromatin structure.
  - Methylation: is performed on lysine, arginine and proline residues. It does not change the charge of histone protein but alters the affinity of the genome transcription factors.
  - Acetylation: is associated with the opening form of the chromatin and the onset of transcription, while deacetylation does the opposite by closing the transcription factor binding sites [30].
- MicroRNAs (miRNAs) are a class of non-coding RNAs, about 19–24 nucleotides long that can bind to the 3′UTR region of target mRNA preventing the translation of mRNA and protein production. They play critical roles in regulating cell functions of the cells and can be the cause of many diseases. For instance, in several cancer types, the amount of miR-101 decreases, resulting in an increase in the expression of the regulated methyl transferase which in turn increases the methylations in the tumor suppressor genes, eventually increasing incidence of cancers. In addition, some miRNAs are used as cancer diagnosis biomarkers and determinants of cancer prognosis [31].

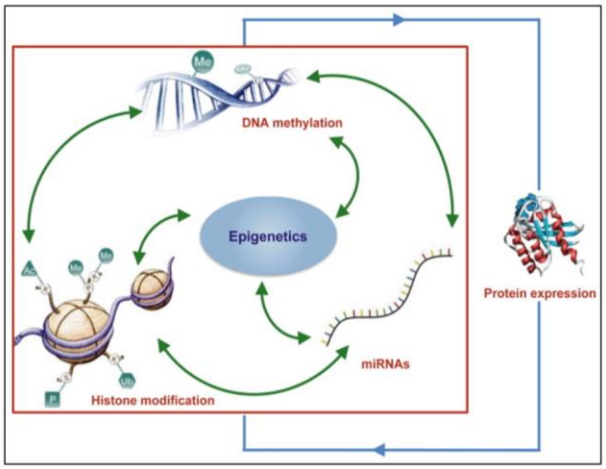


Figure 5: The main areas of epigenetics [32].Main areas of epigenetics are DNA methylation and histone modifications (acetylation, methylation, phospholylation, ubiquitylation) and micro-RNA based mechanisms. These three processes are distinct but are interrelated and control gene expression.

1.2.2 THE HALLMARKS OF CANCER

The hallmarks of cancer are classified into six biological categories according to the multistep development of human tumors (**figure 6**) [1].

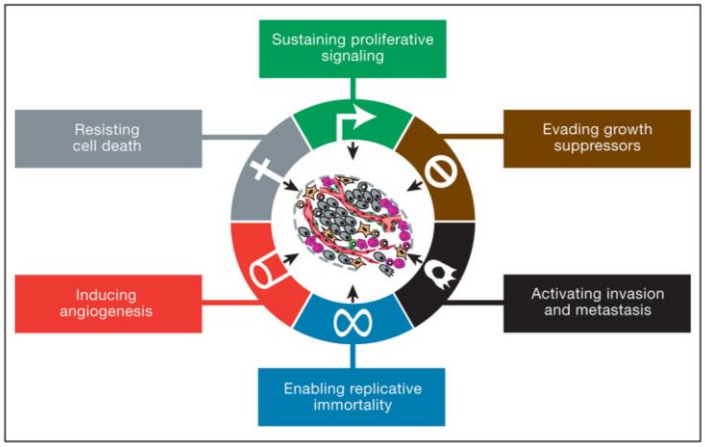


Figure 6. The Hallmarks of Cancer [1]. This illustration encompasses the six hallmark capabilities originally proposed in our 2000 perspective. The past decade has witnessed remarkable progress toward understanding the mechanistic underpinnings of each hallmark.

1.2.2.1 SUSTAINING PROLIFERATIVE SIGNALING

Normal tissues control the production and release of growth signals that regulate the cell growth and-division cycle, thereby ensuring a homeostasis of cell number and the maintenance of normal tissue function. Cancer cells, deregulate these signals and become masters of their own fate. Upon binding of growth factors to the cell-surface receptors, typically containing intracellular tyrosine kinase domains, the signal is propagated by intracellular signaling pathways that control progression through the cell cycle and growth. However, these signals influence other cell-biological properties, such as cell survival and metabolism [33]. Cancer cells adapt the capability to sustain proliferative signaling in an autocrine proliferative stimulation, they can produce growth factor ligands, to which they can respond by expressing the conjugate receptors. Alternatively, cancer cells may stimulate normal cells in the supporting tumor-associated stroma, which in turn supply the cancer cells with various growth factors [34]. Cancer cells can also increase the levels of receptor proteins displayed at their surface, which renders them hyperresponsive to the limiting amounts of growth factor ligand.

Cancer cells induce the constitutive activation of signaling pathways operating downstream of these receptors, which are growth factor independent to bypass the stimulation of these pathways by ligand-mediated receptor activation. An example is the Ras signal transducer.

1.2.2.2 EVADING GROWTH SUPPRESSORS

Cancer cells hold up powerful programs that negatively regulate cell proliferation; they depend on the actions of tumor suppressor genes. Many tumor suppressors operate in various ways to limit cell growth and proliferation.

Two typical tumor suppressors encode the RB (retinoblastoma-associated) and P53 proteins; they govern the decisions of cells to proliferate or, to activate senescence and apoptotic programs. The main difference between P53 and RB is that the later transduces extracellular growth inhibitory signals whereas P53 receives inputs from stress that function within the cell. Cancer cells with defects in RB pathway miss the services of a critical gatekeeper of cell-cycle progression permitting the persistent of cell proliferation. In the other hand, P53 can trigger in response to genomic damage to induce apoptosis [35].

1.2.2.3 RESISTING CELL DEATH

Various physiologic stresses in cancer cells impair apoptosis to induce tumorigenesis. Among these apoptosis-inducing stresses are the signaling imbalances resulting from high levels of oncogene expression and DNA damage associated with hyperproliferation. Apoptosis attenuation in those tumors enhances the states of high-grade malignancy and resistance to therapy [36].

Both extrinsic and intrinsic apoptosis exist. Extrinsic apoptosis is induced by the expression of death receptor ligands or by the removal of dependence receptor ligands. CASP8 and CASP10 are involved in death receptor-mediated extrinsic apoptosis, whereas CASP9 starts the withdrawal of dependence receptor ligand-mediated extrinsic apoptosis. FADD (Fas-associated via death domain), is activated upon binding to cell death receptors responding to their ligands and by turn activates Pro-CASP8 and pro-CASP10 that are enzymatically inactive.

DNA damage, hypoxia and metabolic stress can induce intrinsic apoptosis, which leads to the release of mitochondrial proteins into the cytosol where they interact with APAF1 to recruits pro-CASP9 to form the apoptosome [37].

1.2.2.4 ENABLING REPLICATIVE IMMORTALITY

Cancer cells require unlimited replicative potential to be able generate macroscopic tumors. They have the capability to bypass two distinct barriers to proliferation: senescence, which is an irreversible entrance into a non-proliferative but viable state, and crisis, which involves cell death. This transition is termed as immortalization, consist of the ability to proliferate in culture without evidence of either senescence or crisis. Immortalization involves the telomeres, the protecting the ends of chromosomes to reach unlimited proliferation [38].

The telomeres usually shorten progressively in non-immortalized cells, losing the ability to protect the ends of chromosomal DNAs and triggering entrance into crisis.

Telomerase, is a DNA polymerase that adds telomere repeat to the ends of telomeric DNA, is almost absent in non-immortalized cells but highly expressed in 90% of spontaneously immortalized cells, including human cancer cells.

The eventual immortalization of cells that proceed to form tumors is due to their ability to maintain telomeric DNA at lengths enough to avoid triggering senescence or apoptosis.

1.2.2.5 INDUCING ANGIOGENESIS

Tumors require nutrients and oxygen supply as well as the need to evacuate metabolic wastes and carbon dioxide, as normal cells do. The new vasculature associated with tumors, generated by the process of angiogenesis, addresses these needs.

During tumorigenesis, an ‘‘angiogenic switch’’ is activated, causing normally quiescent vasculature to continually give new vessels to sustain expanding neoplastic growths [39].

These angiogenic regulators include signaling proteins that bind to the cell surface receptors displayed by vascular endothelial cells. The well-known types of angiogenesis inducers are vascular endothelial growth factor-A (VEGF-A) and inhibitors are thrombospondin-1 (TSP-1).

The VEGF-A gene encodes ligands that induce new blood vessel growth during embryonic and postnatal development and maintain the homeostatic survival of endothelial cells both in physiological and pathological situations. VEGF signaling is regulated via three receptor tyrosine kinases (VEGFR-1–3) at multiple levels thus, VEGF gene expression could be upregulated both by hypoxia and by oncogene signaling [40].

TSP-1, a key counterbalance in the angiogenic switch, binds transmembrane receptors on the endothelial cells surface, thereby evokes suppressive signals to reverse proangiogenic stimuli [41].

1.2.2.6 ACTIVATING INVASION AND METASTASIS

As carcinomas arise from epithelial tissue, they progressed to higher pathological grades of malignancy, reflected in invasion and metastasis.

The associated cancer cells typically develop changes in their shape and in their adherence to other cells and to the extracellular matrix (ECM). The best characterized alteration involves the loss of E-cadherin, an adhesion molecule that helps to assemble epithelial cell sheets. The increased expression of E-cadherin counteracts invasion and metastasis, whereas its reduction favors these phenotypes [42].

In contrast, adhesion molecules associated with the cell migrations are often upregulated. For instance, N-cadherin, normally expressed in migrating neurons and mesenchymal cells during organogenesis, is found to be upregulated in many invasive carcinoma cells [43].

The multistep process of invasion and metastasis consist of a succession of cell-biologic changes, it begins with local invasion, followed by the intravasation by cancer cells into nearby blood and

lymphatic vessels to be carried to distant tissues where these cells extravasate from the lumina of such vessels, leading to the formation of small nodules of cancer cells, and finally the growth into macroscopic tumors [43].

1.3 PIN1

Pin1 is a member of the parvulin family and homologues, are the unique peptidylprolyl isomerase (PPIase) that recognizes the phosphorylated Ser/Thr-Pro motifs and catalyzes their isomerization.

Pin1 consists of two domains structures, an N-terminal domain called WW domain (referring to two invariant Trp residues) and a C-terminal domain called PPIase domain. Physiological conditions firmly regulate Pin1 localization and function. Beside some exceptions such as in neurons in neuronal differentiation, Pin1 is expressed in cells with proliferative potential, in normal human cells. However, Pin1 is overexpressed in several human cancers and downregulated during aging [44]. Pin1, acts as a molecular timer, regulating phosphorylation-dependent or phosphorylation-independent based on specific substrates, controls the amplitude and the duration of a cellular process therefore, Pin1 serves as an ON/OFF switch in cells, which has double role in different conditions to promote apoptotic or proliferative mechanisms [45].

PIN1 activates more than 40 oncogenes and inactivates 20 tumor suppressors. It is unclear whether PIN1 is regulated differently in different cancer types and how such mechanisms are coordinated to contribute to disease development and progression. However, Pin1 substrates containing p-Ser/Thr-Pro motifs, can be important cell cycle regulators, as well as oncogenic and tumor suppressor proteins [44].

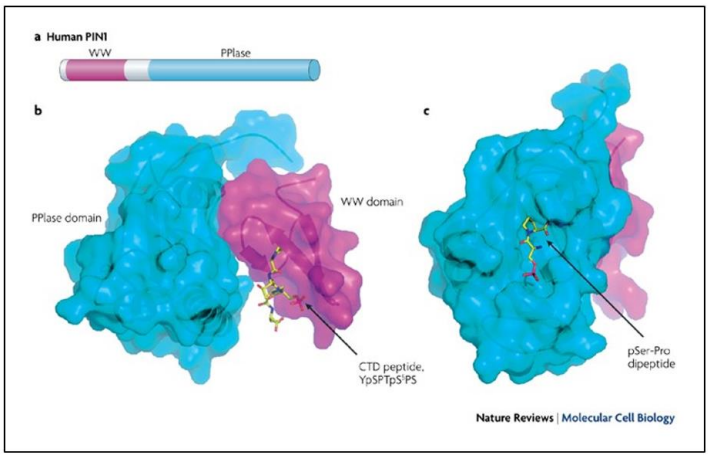


Figure 7: Structural basis for the unique specificity of PIN1 towards specific pSer/Thr-Pro motifs [46]. a. The domain architecture of human PIN1which contains an N-terminal WW domain, which mediates binding to specific pSer/Thr-Pro motifs, and a C-terminal peptidyl-prolyl cis/trans isomerase (PPIase) domain that catalyses isomerization of specific pSer/Thr-Pro motifs in the substrate. b. X-ray structures of PIN1 in a complex with a C-terminal domain (CTD) peptide (YpSPTpS5PS) in the WW domain. Ser16, Arg17 and Tyr23 in the WW domain are responsible for binding the phosphate of pSer5 (the fifth residue in the peptide) and the aromatic rings of Tyr23 and Trp34 form an aromatic clamp, which accommodates the ring atoms of Pro6, the sixth residue49. c. X-ray structure of PIN1 in complex with a pSer-Pro dipeptide modelled in the PPIase domain10. A set of conserved catalytic residues in all PIN1-type PPIases project outward from the barrel structure and define the binding pocket for the proline and the peptide bond that undergoes cis/trans isomerization. The side chains of Lys63, Arg68 and Arg69 form a basic cluster that sequesters pSer in the substrate. Lys63 is conserved in PIN1- and parvulin-type PPIases and is involved in basic catalysis. In contrast, Arg68 and Arg69 are conserved only in PIN1-type PPIases and are responsible for the unique phosphorylation specificity of PIN1-type PPIases.

1.3.1 PIN1 AS AN ONCOGENE OR CONDITIONAL TUMOR SUPPRESSOR

PIN1 is widely overexpressed in different types of cancers, it was found to be involved in prostate, breast, oral squamous carcinomas and other cancers [47]. This overexpression results in disrupting cell cycle coordination which leads to chromosomal instability and tumorigenesis. However, Pin1 is not an oncogene by itself but it was found to be upregulated in different types of human cancers, it promotes cancer by upregulating the expression of some oncoproteins [48].

For example, Pin1 regulates the expression of the oncoprotein cyclin D1 at both transcriptional and post-transcriptional levels. First, the tumor suppressor APC binds β-catenin thereby inhibits the transcription of the oncogene cyclin D1. In cooperation with oncogenic Ras signaling Pin1

regulates the expression of cyclin D1 binds and isomerizes β-catenin p-Ser246-Pro motif inhibiting the interaction between β-catenin and APC, leading to aberrant accumulation of β-catenin in the nucleus which enhances the activation of cyclin D1 that leads to cell transformation [49].

Moreover, after GSK-3β phosphorylation of cyclin D1 at the p-Thr286-Pro motif, Pin1 binds cyclin D1 and catalyzes its isomerization thus inhibits its interaction with CRM1, increasing stabilization and an accumulation of cyclin D1 in the nucleus [50].

Another example, Pin1 recognizes and binds p-Ser63/73-Pro motifs in c-Jun after its phosphorylation by c-Jun N-terminal kinases (JNKs) triggered by Ras signaling, therefore Pin1 regulates the activity of c-Jun, increasing transcriptional activity of c-Jun towards the cyclin D1 promoter.

Furthermore, Pin1 increases the stability or the activation by binding of several other oncogenic proteins, including NF-κB, Akt, Stat3, and Mcl-1 [51].

In addition, Pin1 controls tumor cell proliferation by altering the phosphorylation of the retinoblastoma protein (pRb) allowing its interaction with CDK/cyclin complexes in mid/late G1, resulting in uncontrolled cell proliferation.

Altogether, these results pose Pin1 as a tumor promoter thus might be an important therapeutic target.

On the other hand, Pin1 plays a crucial role as a conditional tumor suppressor.

Pin1 induces apoptosis by enhancing the function and increasing the protein stability and transcriptional activity of p53 and p73 in stress conditions, suggesting that it may have some tumor suppressive activities in cells.

Various studies support the role PIN1 may have in controlling the degradation of key oncogenic proteins such as MYC, PML-RARα, Bcl-6 and cyclin E [52].

Pin1 participates in the pathway that regulates the ubiquitin-dependent proteolysis of Myc through the Ras-mediated signaling cascade, where MAPK is activated and phosphorylates Myc on Ser62, stabilizing the protein. The phosphorylation induces a second phosphorylation of Myc on a Thr58, by glycogen synthase kinase 3 (GSK3). Pin1 binds to the double-phosphorylated Myc and facilitates its dephosphorylation of Ser62 by protein phosphatase 2 (PP2A), which promote ubiquitylation and degradation of Myc by the proteasome.

Additionally, upon DNA damage, the kinase ATM promotes Bcl-6 phosphorylation, and

consequently its interaction with Pin1 leading to its degradation by the ubiquitin-proteasome system [53].

Finally, the aberrant expression of cyclin E affects cell cycle and induces oncogenesis. Ser380 and Ser384 Cyclin E motifs are phosphorylated by Cdk2 and GSK-3β at Ser380 respectively. Pin1 binds p-Ser384 and regulates cyclin E, it promotes cyclin E protein degradation during cell cycle progression, whereas the absence of Pin1 results in an accumulation of cyclin E leading to a delayed G0/G1-S phase progression [54].

1.3.2 REGULATION OF PIN1

Cell growth, proliferation and survival are controlled by extra- and intracellular stimuli, which are regulated by the activation of signaling events such as conformational changes in protein kinase and their substrate in response to protein phosphorylation. These conformational changes regulate protein activities at specific time and location depending on different physiologic conditions and diseases.

After transcription, *PIN1* mRNA stability is inhibited by microRNAs (miRNAs).

For instance, miR200c binds directly to a conserved region in the 3′ -untranslated region (UTR) of Pin1. Mutations in this region can prohibit the repressing effect of miR200c on Pin1 transcription. miRNA-200b regulates programmed cell death by directly targeting the 3′ UTR of Pin1 mRNA and regulating Pin1 expression. Downregulating miRNA-200b promotes cancer cells survival [55].

miR-296-5p has a conserved binding site in the Pin1 3' (UTR), it plays a tumor-suppressive role by targeting Pin1 [56].

1.3.2.1 PIN1 POSTTRANSLATIONAL REGULATIONS

Post-translational modifications manage Pin1 expression, for instance Pin1 phosphorylation on Ser16 in WW domain suppresses its ability to interact with its substrate; three different proteins can act on this site to phosphorylate it; protein kinase A (PKA;)ribosomal S6 kinase 2 (RSK2) and Aurora kinase A (AURKA) [51]. Some reports evidenced that, the phosphorylation of WW domain on Ser16 site in Pin1 is fundamental, it mediates Pin1 substrate interaction and subcellular localization. Pin1 is phosphorylated in cell cycle-regulated mode , and interestingly, Pin1 is found to be dephosphorylated in breast cancer cells [51]. A mutation of Pin1in Ser16

converting to Glu16 ($Pin1^{S16E}$), abolished the ability of Pin1 to bind mitotic phosphoproteins by its WW domain. However, another mutation converting the same Ser16 site to Ala16 ($Pin1^{S16A}$) did not alter Pin1 binding activity and it was able to bind almost all substrates of Pin1inducing mitotic block and apoptosis [57].

In addition, the inhibition Pin1 activity can be also favored by its phosphorylation at Ser71 by the tumor suppressor gene, death-associated protein kinase 1 (DAPK1). Phosphorylated, inactive Pin1, inhibits it ability to induce cellular transformation [58].

On the other hand, Pin1 phosphorylation on Ser65 by PLK1 polo-like kinase or phosphorylation on Ser138 by Mixed-lineage kinase 3 (MLK3) increase PIN1 catalytic activity and nuclear translocation [58]. Pin1 phosphorylation on Ser65 by PLK1 induces its deubiquitylation and stabilization. Interestingly, expression of a dominant negative form of Plk1 or by transfection of small interfering RNA targeted to Plk1 to inhibit its activity, enhances the ubiquitination of Pin1 and subsequently reduces the amount of Pin1 in human cancer cells [59].

Furthermore, sumoylation of Lys6 in the WW domain and Lys63 in the PPIase domain respectively can abolish Pin1. However, desumoylation of these two domains by SUMO1/sentrin specific peptidase 1 (SENP1) increases PIN1 substrate-binding and oncogenic function.

Furthermore, oxidative modification on Cys113 in the PIN1 active site inhibits PIN1 enzymatic activity and inactivates its ability to inhibit the development of Alzheimer disease [51] and deacetylation of Pin1 on K13 and K46 that promotes its function (WO 2016011265 A2).

1.3.2.2 PIN1 A POSSIBLE BIOMARKER OF CANCER

Pin1 is found to be overexpressed in some human malignancies. Several oncogenic pathways are enhanced by the upregulation of Pin1. They include non-small cell lung cancer hepatocellular carcinoma induced by hepatitis B virus (HBV), Burkitt lymphoma induced by MYC75 activation and T cell acute lymphoblastic leukemia induced by NOTCH3 [44].

Furthermore, Pin1 is a target for E2F family of transcription factors (E2F1, E2F2 and E2F3), which essentially induces *Neu/Ras* transformation of mammary epithelial cells; these transcription factors interact with the *Pin1* promoter region for E2F binding sites and increase PIN1 protein levels [47].

PIN1 is identified as a target of the oncogenic C/EBPα-p30 protein. The last is generated by mutations in *CEBPA*, it is a dominant negative isoform of the tumour suppressor C/EBPα that is

found in approximately 10% of patients with acute myeloid leukemia. This C/EBPα-p30 recruits E2F family proteins to bind to the *PIN1* promoter [60].However, the expression of Pin1 that is regulated in response to growth factors by E2F-mediated transcriptional regulation is suppressed by the tumor suppressor gene BRCA1.

The relationship between Pin1 expression and the clinical outcome of cancer patients suggested that Pin1 protein expression is positively correlated to clinical staging, by comparing different clinical features. Interestingly, higher probability of recurrence of prostate cancer was detected in patients with a higher Pin1 expression compared to their counterparts with low Pin1 expression, after radical prostatectomy. Moreover, the risk of earlier reoccurrence, was 4 times higher patients with high Pin1 expression than those with low Pin1 expression; and 8.1 times more in patients with a very high level than a low Pin1 expresser. Pin1 is also a cofactor for androgen receptor (AR) that was found to play an important role in prostate cancer by allowing aberrant expression of several genes involved in cancer initiation, progression and metastasis [61]. In addition, Pin1 expression can be used as excellent prognostic marker as well as good clinicopathological parameter for preoperative setting to assist in choice of treatment in prostate cancer.

Pin1 silencing in lymphomas delayed the disease progression in mice where Pin1 was essential for Myc-induced tumorigenesis [62].

The overexpression of Pin1 was found to disrupts the coordination between DNA synthesis and centrosome duplication during the cell cycle which correlated to tumorigenesis *in vitro,* in transgenic mice and in human cancer tissues. Indeed, PIN1 knockdown are highly resistant to breast cancer cell growth *in vitro* and *in vivo*. The difference of expression of Pin1 between normal breast cells and breast cancer cells revealed its pivotal role in breast cancer, PIN1 expression is approximately 5 times higher in non- cancer stem cells tumor cells (CSCs) and more than 30 times higher in CSCs [63].

The oncogenic potential of Pin1 in lung cells, was determined by studying the effect of its overexpression in Gejiu Lung Carcinoma-82 cells (Glc82), and its downregulation by RNA interference in H1299 cells (human non-small cell carcinoma cell line that do not express tumor suppressor p53). High expression of Pin1 correlates with poor survival, showing that high expression of Pin1 is an independent prognostic factor. As in clinical findings, overexpression of Pin1 in Glc82 cells was associated with cell growth and tumorigenicity in nude mice [64].

In HBV-related Hepatocellular carcinoma, Pin1 overexpression increased the protein stability of HBx. Cell lines expressing Pin1 and HBx showed an interactive increase in cellular proliferation, in comparison with cells expressing Pin1 or HBx alone. In addition, the simultaneous expression of Pin1 and HBx in the nontumorigenic human hepatocyte cell line induced synergistic increase in tumor growth, whereas the suppression of Pin1 expression in human hepatoma cells HBx-enhanced tumor growth was inhibited [65].

1.4 TREATMENT

The increasing knowledge of molecular and cancer biology has influenced cancer treatment paradigms. Both classification and treatment of cancer were according to organs of origin or simplistic histo-morphologic features. After using four platinum-based chemotherapy in lung cancer, it was found that cancer treatment based on cytotoxic chemotherapies in unselected patients, reached its therapeutic plateau. Therefore, the need of molecularly targeted therapies was needed.

New technology for the acquisition of tumor molecular profiling and for the discovery of predictive molecular targets are needed. Revolution in cancer treatment was based first on genotype-directed precision oncology and second on targeting components of the tumor microenvironment [66].

1.4.1 CURRENT CANCER TREATMENTS

Many types of cancer treatment exist, depending on the type and the stage of cancer.

Some cancer patients will have only one treatment, but most people have a combination of treatments, such as surgery with chemotherapy and/or radiation therapy (**figure 8**).

(https://www.cancer.gov/about-cancer/treatment/types)

Treatment strategies of cancer include:

- Surgery is the procedure of removing cancer from the body.

There are many types of surgeries which differ based on the purpose of the surgery, the part of the body that requires surgery and the size of tissue to be removed. It may be open or minimally invasive:

- In open surgery, one large cut is made to remove the tumor, some healthy tissue, and possibly some nearby lymph nodes.
- In minimally invasive surgery, few small cuts are made instead of one large one. Inserting a long, thin tube with a tiny camera into one of the small cuts to project images from the inside of the body onto a monitor, allows the surgeon to see and remove the tumor.

- Radiation therapy or radiotherapy uses high doses of radiation to kill cancer cells and shrink tumors.

At high doses, radiation therapy kills cancer cells or reduces their growth ability by causing DNA damage consequently leading to cell death. However, it takes from days to weeks after treatment for the DNA to become damaged enough to induce cancer cells to die.

The type of radiation therapy depends on many factors, including: the type of cancer, the size of the tumor, the location in the body, the vicinity of the tumor to normal tissues, age and other medical and health conditions.

- Internal radiation therapy consists of putting a source of radiation is put inside the body. The radiation source can be solid or liquid. A solid source is called brachytherapy where a kind of capsules containing a radiation source are placed in the body or near the tumor. However, it treats only a specific part of your body.
- Internal radiation therapy, also called systemic therapy, consists of delivering a liquid that travels in the blood to tissues throughout the body. It could be delivered by swallowing, intravenous, or through an injection.

- Chemotherapy works by stopping or slowing the growth of cancer cells, which have high growth and division rates.

Chemotherapy types include:

- Alkylating agents are the oldest group of chemotherapeutics that are used nowadays. They are

so named because of their ability to bind covalently via their alkyl group to molecules, such proteins, DNA and RNA. Alkylating agents can work in different points in the cell cycle so are known as cell cycle-independent drugs. Cisplatin are subtypes of alkylating agents. Cisplatin and derivatives include cisplatin, carboplatin and oxaliplatin. They affect cell function by covalent bonding with the amino, phosphate, carboxyl and sulfhydryl groups in biologically important molecules [67].

- Anti-metabolite are a group of molecules that inhibit DNA and RNA synthesis. Mainly, they have a similar structure to the nucleotides, the building blocks of DNA and RNA. However, they have different chemical groups, they work by either blocking the enzymes involved in DNA synthesis or becoming incorporated into DNA or RNA. Unlike alkylating agents, they are cell cycle dependent, they work only during the S-phase of the cell cycle. The anti-folates include methotrexate and pemetrexed. Methotrexate inhibits the dihydrofolate reductase, the enzyme that regenerates the tetrahydrofolate required for purine (DNA bases) synthesis. Pemetrexed also affects purine and pyrimidine production and inhibits DNA synthesis. Fluorouracil is a type of fluoropyrimidines, a nucleobase analogue that becomes incorporated into RNA and inhibits the enzyme thymidylate synthase and leads to cell death [68].
- Anti-microtubule agents are plant-deriving chemicals that block microtubule function during cell division. These drugs can affect blood vessel formation during tumorigenesis. They are cell cycle- specific, they bind to the tubulin microtubule during the S-phase and inhibit microtubule formation during M-phase. Paclitaxel is a subtype of Taxanes, that prevent the cell cycle at G2-M phase. The main difficulty in Taxanes formulation as medicines is that they are poorly soluble in water [69].
- Topoisomerase inhibitors are drugs that affect the activity of topoisomerase I and topoisomerase II enzymes that unwind or produce single- or double-strand breaks into DNA, to reduce the tension in the DNA strand. This allows the normal unwinding of DNA to proceed with replication or transcription. Irinotecan and topotecan that inhibit these two enzymes, preventing DNA replication and transcription, causes DNA strand breaks, and leads to programmed cell death. Doxorubicin is a common topoisomerase II inhibitor [70].

- Immunotherapy is a type of cancer treatment that helps the immune system to fight cancer.

Immunotherapy is a type of biological therapy that uses substances made from living organisms to treat cancer.

Normally, the immune system detects and destroys abnormal cells and may prevent the growth of many cancers. Indeed, immune cells are sometimes found in tumor zone. These cells, called tumor-infiltrating lymphocytes, are biomarker of the immune system response to the tumor. Patients whose tumors contain infiltrating lymphocytes have better prognosis.

However, cancer cells have ways to be protected and to avoid destruction by the immune system by inducing genetic changes to be less visible to the immune system and expressing proteins on their surface that inhibit the immune cells.

Several types of immunotherapy are used to treat cancer including:

- Immune checkpoint inhibitors**,** are drugs that block immune checkpoints. These checkpoints are normally a part of the immune system that control the immune responses from being too strong. Thus, their inhibition allows immune cells to respond more strongly to cancer.
- T-cell transfer therapy**,** is a treatment that boosts the natural ability of the T cells to fight cancer. It consists of taking the most active immune cells from the tumor, performing some modifications to become more potent against cancer cells, grown in large batches, and then put back into the body through a needle in a vein.
- Monoclonal antibodies, are immune system proteins created in the lab against specific targets on cancer cells. These monoclonal antibodies label cancer cells to be destroyed by the immune system.
- Treatment vaccines**,** work by boosting the immune system's response to cancer cells. They are different from the ones that help prevent disease.
- Immune system modulators**,** enhance the body's immune response against cancer.

- Targeted therapy is the foundation of precision medicine (an approach to patient care that allows treatments selection based on a genetic understanding of their disease.)

It targets proteins that control cancer cells growth, division and spread.

The types of targeted therapy include:

- Small-molecule drugs that enter cells easily to reach their targets inside cells.
- Monoclonal antibodies are designed to attach to specific targets found on cancer cells. They can

mark cancer cells to be destroyed by the immune system or can directly stop cancer cells from growing or cause them to self-destruct.

- Hormone therapy slows or stops the growth of cancer that rely on hormones to grow. It is also known as endocrine therapy.

Hormone therapy can block the body's ability to produce hormones or can interfere with the normal function of hormones in the body.
Hormone therapy is used to:
- Treat cancer slowing its growth and reducing the chance that cancer relapses.
- Ease cancer symptoms especially in men with prostate cancer who are not able to have surgery or radiotherapy.

- Stem cells transplants restore blood-forming stem cells in patients who have had theirs destroyed by the very high doses of chemotherapy or radiation therapy.

Blood-forming stem cells are important because they grow into the various types of blood cells. In a stem cell transplant, healthy blood-forming stem cells are delivered intravenously to enter your bloodstream, and travel to reach the marrow and substitute the damaged cells.
Transplants can be:
- Autologous, the stem cells come from the patient.
- Allogeneic, the stem cells come from someone else such as a blood relative or someone who is not related.
- Syngeneic, the stem cells come from an identical twin.

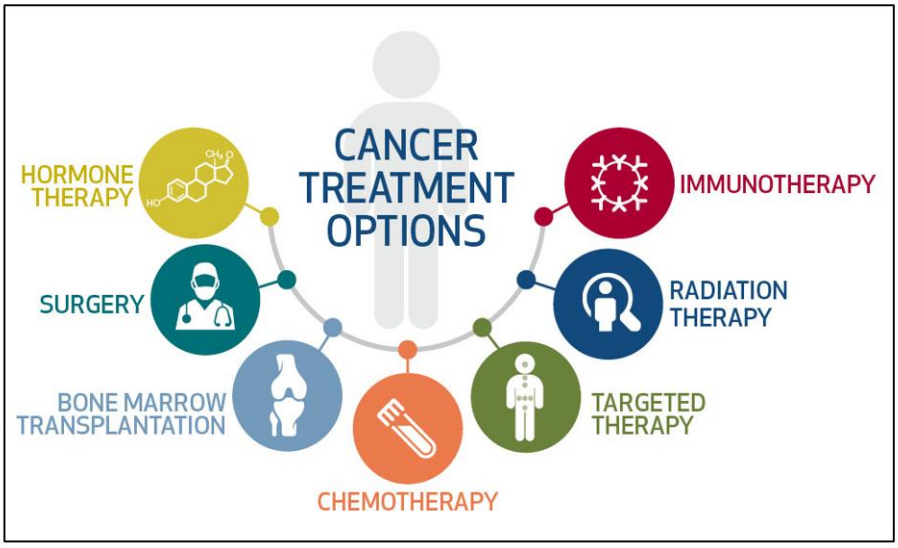


Figure 8: CANCER TREATMENT OPTIONS. (From: https://www.indiaehs.com/cancer-treatment-in-india/)

1.4.2 INTRINSIC AND ACQUIRED DRUG RESISTANCE

Drug resistance can be categorized as intrinsic or acquired resistance based on the time when it is developed.

1.4.2.1 INTRINSIC RESISTANCE

Intrinsic resistance represents the innate resistance that exists before the patient is exposed to drugs, which causes reduced efficacy of the drug treatment. It can be caused by:

- Pre-existing genetic mutations in the majority of tumors causing decreased responsiveness of cancer cells and may also lead to relapse. For example, the high expression of *HER2* gene induces the upregulation of transcription factor Snail, causing a morphological change like epithelial-mesenchymal transition which makes cancer cells more resistant.
- Heterogeneity in tumors where pre-existed insensitive subpopulations like cancer stem cells are selected after drug treatment, leading to relapse in later stages of treatment. Cancer stem cells (CSCs) are a subpopulation in tumors capable of self-renewal and

differentiation and participate in tumor initiation and progression. They are involved in resistance to chemotherapeutic drugs in different cancer types, including leukemia, glioblastoma and pancreatic cancer.

- Activation of intrinsic pathways for defense against environmental anti-cancer drug toxins. For instance, the ATP binding cassette (ABC) transporter mediate drug efflux and glutathione (GSH)/glutathione S-transferase system, which normally reduce drug accumulation or detoxify drug treated cancer cells respectively.

1.4.2.2 ACQUIRED RESISTANCE

Acquired resistance is the gradual reduction of anticancer efficacy as a result of drug treatment. Acquired resistance can be a consequence of:

- Activation of second proto-oncogene to becomes the new driver gene. Novel gene mutations appeared in relapse tumors that were not found in primary tumors due to DNA damage in cancer cells that might increase probability of the emergence of new mutations after the treatment.
- Mutations or altered expression levels of the drug targets. Cancer cells can acquire resistance to targeted drugs after mutating the genes encoding target proteins or changing their expression levels. For example, the tyrosine kinase inhibitor (TKI) imatinib targeting BCR-ABL, commonly used in chronic myelogenous leukemia treatment induced resistance or relapse after treatment in approximately 20%~30% of patients, due to a point mutation of T315I of the fusion tyrosine kinase protein.
- Changes in tumor microenvironment (TME) after treatment. During tumor progression, a crosstalk exists between tumor cells and their microenvironment. For example, exosomes are released by cancer and stromal cells, carrying certain miRNAs to be used by cancer cells and tumor-associated macrophages to communicate with each other. This exchange of miRNAs between tumor cells and stromal cells in can promote drug resistance [71].

The mechanisms of intrinsic and acquired resistance can co-exist during tumor progression and treatment. After acquired drug resistance development, therapeutic schemes should be updated

accordingly to delay drug resistance. They should aim at slowing down or stop tumor growth without inducing acquired drug resistance. Genomic and other biochemical screening should before the drug treatment plan can be helpful to avoid potential pre-existing drug resistance.

1.5 NANOPARTICLES

Nanoparticles (NPs) are very tiny materials with size ranges from 1 to 100 nm. They are classified based on their properties, shapes or sizes. NPs' unique physical and chemical properties are due to their high surface area and nanoscale size. They are suitable candidates for various commercial and domestic applications including imaging, medical applications, energy-based research, and environmental applications.

1.5.1 CLASSIFICATION OF NPs

NPs are classified based on their morphology, size physical and chemical properties.

- Carbon-based NPs: Fullerenes and carbon nanotubules (CNTs) represent two major types of carbon-based NPs. Fullerenes are made of globular hollow cage such as allotropic forms of carbon characterized by their electrical conductivity, electron affinity, high strength, structure, and versatility. CNTs are elongated, tubular structure, 1–2 nm in diameter, predicted as metallic or semiconducting. They structurally resemble to graphite sheet rolling upon itself; the rolled sheets can be single (SWNTs), double (DWNTs) or multiple (MWNTs), walled carbon nanotubes. Due to their unique physical, chemical and mechanical characteristics, these materials are used for many commercial applications such as fillers and efficient gas adsorbents for environmental remediation.

- Metal NPs: purely made of the metallic precursors. These NPs possess unique optoelectrical properties. NPs of the alkali and noble metals including Cu, Ag and Au have a broad absorption band in the visible range of the electromagnetic solar spectrum. Metal NPs find applications in many research areas; gold NPs coating is widely used for

in SEM sampling to enhance the electronic stream leading to high quality SEM images.

- Ceramics NPs: are inorganic nonmetallic solids, synthesized via cycles heat and successive cooling. They can be found in different form: dense, amorphous, polycrystalline, porous or hollow forms. These NPs have many applications such as catalysis, photocatalysis, photodegradation of dyes, and imaging applications.

- Semiconductor NPs: possess properties between metals and nonmetals. They possess wide bandgaps and eventually showed significant alteration in their properties with bandgap tuning. They have very important applications in photocatalysis, photo optics and electronic devices.

- Polymeric NPs: are normally organic NPs, they are mostly nanospheres or nanocapsular shaped. The nanospheres are matrix particles whose overall mass is generally solid, and the other molecules are adsorbed at the outer boundary of the spherical surface. In nanocapsular form, the solid mass is encapsulated within the particle completely.

- Lipid-based NPs: contain lipid unit and are effectively used in various biomedical applications. They are spherical with diameter ranging from 10 to 1000 nm and they possess a solid core made of lipid and a matrix contains soluble lipophilic molecules. To stabilize the external core of these NPs, surfactant and emulsifiers are usually used. Various applications of these NPs as drug carriers, in delivery and RNA release in cancer therapy [72].

1.5.2 VARIOUS TYPES OF NANOPARTICLES FOR INTRACELLULAR APPLICATIONS

Different types of nanoparticles (**table 1**) are used as tools for drug delivery, imaging, sensing, and for the understanding of basic biological processes include:

- Liposomes: are the first NP platform. They are applied for gene and drug delivery. They are spherical vesicles containing either single or multiple structure of lipids bilayer that self-assemble in aqueous systems. The advantages of liposomes are:
  - Their abilities to carry and protect many types of biomolecules
  - Biocompatibility
  - Biodegradability.

  Liposomes are used as transfection agents of genetic material into cells. They are also used as therapeutic carriers; hydrophilic compounds are entrapped within the core whereas the hydrophobic drugs are bound to the lipid bilayer itself. Liposomes were conjugated with biocompatible polymers such as polyethylene glycol (PEG) to enhance their stability and circulation half-life *in vivo*.

- Albumin-bound NPs (nab): they use the endogenous albumin pathways to bring the hydrophobic molecules in the bloodstream. They are capable to bind to the hydrophobic molecules in non-covalent reversible binding, bypassing solvent-based toxicities for therapeutics. Abraxane, is a combination of 130-nm nab paclitaxel to be approved by the FDA in 2005 for metastatic breast cancer treatment.

- Polymeric NPs: are synthesized using biocompatible and biodegradable polymers used as therapeutic carriers. They are formulated through block-copolymers of different hydrophobicity that can spontaneously assemble into a core-shell micelle form in an aqueous environment. They were used to encapsulate hydrophilic and hydrophobic small drug molecules, proteins and nucleic acid macromolecules. They allow slow and controlled release of drug at target site, improving drug safety and efficacy. Dendrimers are another type of polymeric NPs, that are regularly branched macromolecules that can be either synthetic or natural elements such as amino acids, sugars and nucleotides. They can be loaded with small molecules in their cores via chemical linkage, hydrogen bond, and or hydrophobic interaction. They can be also as detecting and imaging agents, and therapeutic attachment sites as well.

- Iron oxide NPs: The superparamagnetic iron oxide NP (SPION) consist of an iron oxide core coated with a hydrophilic coat of dextran or other biocompatible compound to increase their stability. The most widely used SPIONs are formed by magnetite (Fe_3O_4) and/or maghemite (γFe_2O_3) which allows them to become magnetized by applicating an external magnet. They have several advantages including decreased toxicity and increased imaging sensitivity. Two currently approved SPIO agents are ferumoxides (120–180 nm) and ferucarbotran (60 nm).

- Quantum dot: are semiconductor particles with less than 10 nm in diameter. They display unique size-dependent electronic and optical properties. Most studied QDs are formed by a cadmium selenide core and a zinc selenide cap. They have a wide absorption spectrum and a narrow band of emission. They have different biochemical specificities; they can be simultaneously excited and detected. They can be widely used as fluorescence imaging tools for applications in biological research such as cell labeling. They are also suitable for biomedical applications such as medical imaging and diagnostics.

- Gold NPs: have many size-and-shape dependent physical and chemical properties, biocompatibility, and easy surface modification. They can strongly improve optical processes and surface-enhanced Raman scattering (SERS). They are useful in many fields including biochemical sensing, bio-imaging, diagnostics, and therapeutic applications. Gold NPs are used in colorimetric arrays as substrates in SERS to enhance Raman scattering, allowing spectroscopic identification of proteins and molecules at the NP surface. Gold NP probes are used in the diagnostics of heart disease and cancer biomarkers. In addition, they transform the absorbed light into heat, consequently they have high potential for infrared phototherapy [73].

Table 1: Examples of various types of nanoparticles being developed for intracellular applications [74].

Type of nanoparticle	**Typical size range/nm**	**Structure and properties**
Inorganic Metals (Au, Ag, Cu)	5–250	• Easy to synthesize over a broad range of sizes and shapes (e.g. spheres, rods, core–shells); robust and functionalizable via thiol-metal chemistry • Surface plasmon resonance; surface enhanced Raman scattering
Iron oxides	5–200	• Typically, magnetite (MxFe3_xO4, M = Mn, Ni, Co, Fe) or maghemite (Fe2O3) • Ferromagnetic or superparamagnetic properties
Quantum dots	3–30	• Typically, II–VI or III–V chalcogenides synthesized as core–shell or alloy nanocrystalline colloids (e.g. CdSe/ZnS, CdTe1_xSex) • Bright, photostable fluorophores with broad absorption and narrow emission; Large two-photon cross section; FRET-donors
Silica	3–100	• Biodegradable; available also in micro- or mesoporous form for encapsulation of dyes and drugs; easily derivatizable with different surface chemistries using silanes
Layered double hydroxide	50–200	• Mg6Al2(CO3)(OH)16_4H2O • Biocompatible and biodegradable in mildly acidic environments; high drug loading capacity
Calcium phosphate	10–100	• Ca5(PO4)3OH • Biodegradable and biocompatible; can be doped with lanthanides or organic fluorophores

Organic Liposomes	Multilayer: 500–5000 Unilayer: 100–500	• Spherical self-closed structures composed of one of more concentric phospholipid bilayers • Biocompatible, can entrap both hydrophobic and hydrophilic moieties; protects payload from external environment • Size and surface functionality can be tuned by adding new ingredients to the lipid mixture prior to synthesis
Polymer micelles	20–200	• Self-assembled spherical micelles composed of amphiphilic block co- or tripolymers containing a hydrophobic core and a hydrophilic corona • Hydrophobic payload can be entrapped in the core • Geometry and functionality can be modularly controlled via the length and composition of the polymer blocks; can be biodegradable
Polymer nanoparticles	50–300	• Linear polymers with payload conjugated to the sidechain; precipitated into colloidal nanoparticles in solution • Controllable size, surface functionality by adjusting polymer length, composition, and synthesis conditions; can be biodegradable
Dendrimers	2–10	• Radially hyperbranched polymers with regular repeat units • High structural and chemical homogeneity; high ligand density and payload capacity per particle; controlled biodegradation • Common dendrimers for biological applications: polyether, polyester, PAMAM

Carbon nanotubes	d = 0.5–3 l = 10 nm to several centimetres	• Single or multi-layered graphene sheets rolled into concentric cylinders • NIR-photoluminescence, strong resonance Raman scattering effects; directional conductivity, high tensile strength • Water-soluble through covalent chemical modification or non-covalent adsorption; ability to translocate cellular membranes via non-endocytosis mechanisms
Viral nanoparticles	25–150	• Self-assembled protein cages with multivalent surface functionalities • Natural ability to internalize and unpack payload within cells

1.5.2.1 METHODS FOR THE SYNTHESIS OF NANOPARTICLES

The preparation of nanoparticles depends on the physical and chemical character of the polymer and the drug to be loaded. The primary methods of nanoparticles synthesis from preformed polymer includes:

- Emulsion-Solvent Evaporation Method: The first step requires emulsification of the polymer solution into an aqueous phase. The second step consists of polymer solvent evaporation, inducing polymer precipitation as nanospheres. The nano particles are collected by ultracentrifugation. To remove stabilizer residue or any free drug, they are washed with distilled water then lyophilized for storage.

- Double Emulsion and Evaporation Method: This technique is employed to encapsulate hydrophilic drug, which involves the addition of aqueous drug solutions to organic polymer solution under vigorous stirring to form water/oil emulsions. This w/o emulsion is added to a second aqueous phase with continuous stirring to form the w/o/w emulsion. The emulsion then proceeded for solvent removal by evaporation and nanoparticles can

be isolated by centrifugation at high speed. The obtained nanoparticles must be thoroughly washed before lyophilization.

- Salting Out Method: based on the separation of a water-soluble solvent from aqueous solution via a salting-out effect. Polymer and drug are initially dissolved in a solvent and subsequently emulsified into an aqueous gel containing the salting out agent (ionic, such as magnesium chloride and calcium chloride, or non- ionic such as sucrose) and a colloidal stabilizer such as polyvinylpyrrolidone or hydroxyethyl cellulose. This oil/water emulsion is diluted with a proper volume of water or aqueous solution to increase the diffusion of solvent into the aqueous phase, thus inducing the formation of nanospheres. This technique used in the preparation of PLA (Pluronic acid), Poly (methacrylic) acids, and Ethyl cellulose nanospheres leads to high efficiency and is easily scaled up. Salting out is not based on an increase of temperature and therefore may be useful to heat sensitive substances that have to be processed. The greatest disadvantages are exclusive application to lipophilic drug and the extensive nanoparticles washing steps.

- Emulsions- Diffusion Method: The encapsulating polymer is dissolved in a partially water-soluble solvent (such as propylene carbonate, benzyl alcohol), and saturated with water to favorize the initial thermodynamic equilibrium of both liquids. Subsequently, the polymer-water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, resulting in solvent diffusion to the external phase and the formation of nanospheres or nanocapsules, according to the oil-to-polymer ratio. At the end, the solvent is eliminated by evaporation or filtration, according to its boiling point. Beside simplicity, the advantages of this technique include high encapsulation efficiencies (generally 70%), no need for homogenization, high batch-to-batch reproducibility, ease of scaleup and narrow size distribution. Disadvantages include the high volumes of water to be eliminated from the suspension and the leakage of water-soluble drug into the saturated-aqueous external phase during emulsification, affecting encapsulation efficiency.

- Solvent Displacement / Precipitation method: consists of the precipitation of a preformed polymer from an organic solution and the diffusion of the organic solvent in the aqueous medium in the presence or absence of surfactant. Drug, polymers and or lipophilic surfactant are dissolved in a semi-polar water miscible solvent such as acetone or ethanol. Then the solution is poured or injected into an aqueous solution containing stabilizer under magnetic stirring. Nanoparticles are formed instantaneously by the rapid solvent diffusion. To remove the solvent from the suspensions requires reduced pressure. The rates of addition of the organic phase into the aqueous phase affect the particles size. A decrease in both particles size and drug entrapment depends on the high rate of mixing of the two-phase [75].

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1.5.3 BIOLOGICAL BARRIERS TO DRUG DELIVERY

Cancer represents the disease where the adequacy of delivery of highly potent chemotherapeutics, but toxic, mechanisms of action affects the efficacious responses and severe morbidity. Although, the contributions in a century of perpetual discovery and development, the available formulations deliver the drugs without localizing specifically at sites of interest. Drug molecules can simply diffuse and release freely throughout the body, resulting in several side effects and limiting the efficacy of proper doses required for an efficient response. This limitations in the delivery to the target sites cause exceptionally high attrition rates of new chemical entities (NCEs) in the therapeutic areas; only 1 in 9 drugs are approved by regulatory authorities due to the lack in efficacy and safety which are the main causes of NCE failure in later-stage clinical trials [76].

Drug delivery platforms based on nanoparticles represent suitable vehicles for overcoming pharmacokinetic limitations in the conventional drug formulations. Nanoparticles, such as liposomes, showed advantageous solubilizing therapeutic cargos, prolonging the circulation times of drugs. The accumulation of long-circulating macromolecules by extravasation through blood vessels in tumors in site-specific localization of chemotherapeutics [77]. Nanoparticle-based drug delivery is emerging as a powerful strategy in several pathologies, ranging from infection to heart failure. In particular, applications of nanoparticle formulations were approved for hepatitis

A, fungal infections, multiple sclerosis and renal disease. For example, administration of liposomal doxorubicin demonstrated an improvement in comparison with the normal formulation, lead to approval by the US Food and Drug Administration as Doxil for the treatment of Kaposi's sarcoma. In addition, nanoparticle albumin-bound paclitaxel (Abraxane) was approved to reduce the side effects of the conventional paclitaxel formulation.

In spite of their potential in increasing drug half-lives and improving drug's accumulation at sites of injury, nanoparticles face a series of biological barriers that limit their site-specific bioavailability, causing failure of proper therapeutic outcomes. Obstacles are summarized in f**igure 9**. include:

- Opsonization and sequestration by the mononuclear phagocyte system (MPS)
- Nonspecific distribution
- Hemorheological/blood vessel flow limitations
- Pressure gradients
- Cellular internalization
- Escape from endosomal and lysosomal compartments
- Drug efflux pumps [78].

These obstacles vary in complexity depending on factors, such as administration route (oral versus intravenous), disease type (cancer or infection) and disease progression state (early- versus late-stage cancers). Researches are trying to bypass these obstacles by incorporating active targeting moieties to enhance the uptake in specific cells or constituent components for stimulus-responsive release such as pH-sensitive, thermosensitive and ultrasound [79].

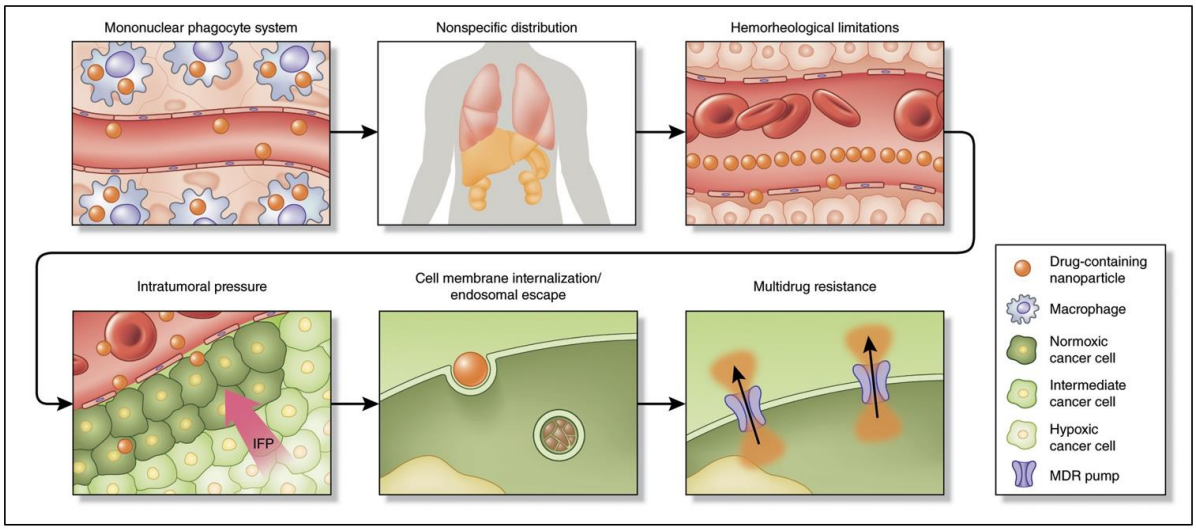

FIGURE 9: Framework of sequential biological barriers to nanoparticle drug delivery [76]. Upon intravenous administration, drug-containing nanoparticles encounter a number of sequential obstacles hindering efficacious, site-specific delivery to tumors. Nanoparticles undergo opsonization and subsequent uptake by resident macrophages of the MPS. This results in high accumulation of nanoparticles in organs, such as the spleen and the liver, contributing to nonspecific distribution of nanotherapeutics to healthy organs. Under normal flow conditions in blood vessels, size and geometry have been shown to vastly influence margination dynamics to vascular walls. Spherical particles of small size migrate in a cell-free layer, at a considerable distance from endothelial surfaces, limiting both active targeting strategies and effective accumulation through passive targeting mechanisms (e.g., EPR). Another substantial barrier to nanoparticle accumulation in tumors is the high intratumoral pressure, resulting from interrupted vasculature, the aggressive nature of cellular growth, fibrosis, a dense extracellular matrix and impaired lymphatics. Cellular internalization and endosomal escape prove to be formidable barriers, with size and surface decoration affecting route of internalization (e.g., clathrin versus caveolin) and intracellular fate. Endosomal compartmentalization of internalized nanoparticles, subjected to a low pH environment and enzymes, proves detrimental to cargo, especially to genetic material. Last but not least, upon entry into the cell, drug efflux pumps that confer therapy resistance expel chemotherapeutics from the cell. IFP, interstitial fluid pressure.

1.5.3.1 NANOPARTICLE RATIONAL DESIGN IMPLEMENTATION FOR OVERCOMING DELIVERY BARRIERS

Understanding delivery barriers, in combination with important advances in materials science at the micro- and nanoscale, has led to the optimization of nanoparticle design parameters.

- Particle size**:** The size of a nanoparticle, which can now be adjusted for precise dimensions for purposes of directing particle distribution *in vivo*. Size controls several biological processes with discrete cut-off size ranges that include circulation half-lives, extravasation through leaky vasculature and macrophage uptake. Particles in the micrometer ranging from 2–5 μm showed an accumulation readily within capillaries of the lungs, providing probably a distinct advantage in targeting one of the predominant sites of metastatic disease. Resident macrophages in the liver, spleen and lungs contribute to particle uptake. Considering the shape and size in nanoparticle design, drives initial internalization and size ultimately determines successful completion of uptake. In particular, nanoparticles averaging ∼100 nm show generally long-lasting in the circulation. Long half-lives in blood increase the capability of nanoparticles to extravasate through fenestrations vasculature in tumors, which range in size from 380–780 nm [80].

- Particle shape: The principle of form follows function has an important impact on nanoparticle architecture, affecting hemorheological dynamics, cellular uptake and *in vivo* destiny. Shape can heavily affect the circulation half-life of a particle. It has been demonstrated that filamentous polymer micelles (filomicelles) have longer-circulating lifetimes reaching more than one week after administration, when compared with spherical counterparts which ranges between 2to 3 days. The ellipsoidal, cylindrical and discoidal particle shapes possess high aspect ratios and minimal regions of curvature, explaining the enhanced accumulation of therapeutics within tumors. Intravenous administration of filomicelles containing paclitaxel was associated with higher accumulation in tumors than spherical micelles [81].

- Surface charge: Nanoparticle surface charge is another design feature that can be optimized to prolong circulation lifetimes and to enhance accumulation at specific sites of interest. Nanoparticles with neutral and negative surface charges have reduced adsorption of serum proteins, leading to a longer circulation half-lives. In contrast, positively charged nanoparticles, showed a higher rate of nonspecific uptake in most of the cells. Indeed, the cationic liposomes preferentially bind and get internalized by tumor-

associated angiogenic endothelial cells compared with normal vasculature, also seen in sites of chronic inflammation. The positively charged particles facilitate endosomal release by hindering degradative effects of the endosomal compartment on drug cargo [76].

- Deformability and degradability: Organs including liver and spleen, have discontinuous endothelia responsible for the filtration of particulates from circulation. Rigid particles with diameters bigger than the cut-off limit of these splenic fenestrations are easily cleared by these organs, demonstrating that 'softer' the nanoparticles, the more prone to deformability and longer the circulation lifetimes and less the accumulation in the spleen. Soft hydrogel particles with various diameters produced using a PRINT (particle replication in nonwetting templates) method, demonstrate long circulation lifetimes exceeding 30h, after intravenous administration. The release of drugs is dependent on the biodegradation kinetics, representing a critical consideration for nanoparticle design. It is important that particles remain stable during circulation to prevent drug accumulation in healthy organs and to maximize their bioavailability at the target site. A combination mixed micelle formulations based on aliphatic polycarbonates, consisted of urea-containing block copolymers and blended with acid-functionalized block copolymers, demonstrated that particle formulations with high kinetic stability accumulate in tumors in a greater extent and more rapidly than formulations with lower kinetic stability [76].

1.6 DOCKING

The concept of molecular docking is to predict the ligand-receptor complex structure-based computation methods (**figure 10**). It could be achieved through sampling conformations of the ligand in the active site of the protein followed by ranking these conformations via a scoring function.

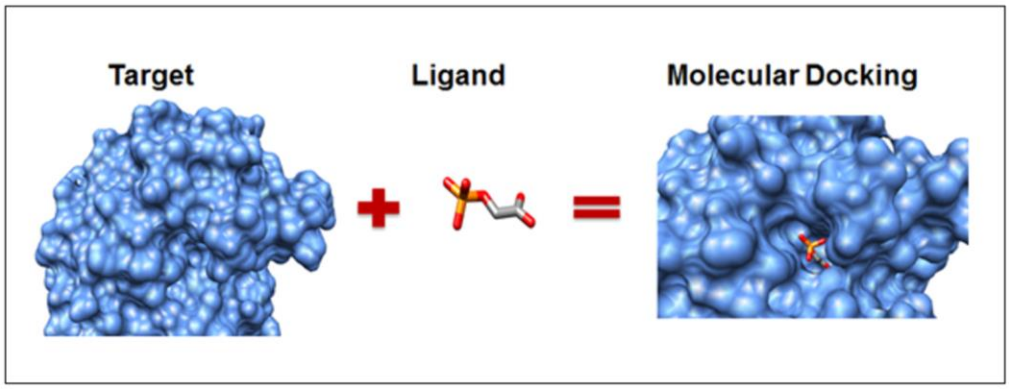

Figure 10: MOLECULAR DOCKING Molecular docking is one of the most frequently used techniques in structure-based drug design (SBDD), due to its ability to predict the binding conformation of small molecule ligands to the suitable target binding site (From: https://www.creative-proteomics.com/).

1.6.1 THE MOLECULAR DOCKING

The complementarity between the human genome project that resulted in an important number of new therapeutic targets for drug discovery, with the high-throughput protein purification, crystallography and nuclear magnetic resonance spectroscopy techniques that contributed to many structural details of proteins and protein–interactors, allowed the computational strategies to perform all aspects of actual drug discovery, including the virtual screening (VS) techniques for hit identification and methods for lead optimization [82]. In comparison to traditional experimental high-throughput screening (HTS), the VS has more significant advantages being a more direct and rational drug discovery approach in addition to its low cost and effective screening.

VS methods can be either ligand-based or structure-based methods.

- The ligand-based method is applied when a set of active ligand molecules is known, and few structural information is available for the targets. It includes pharmacophore modeling and quantitative structure activity relationship (QSAR) methods.
- The structure-based method is the most common and has been widely used ever since the early 1980s [83].

Different algorithms were developed to produce programs to perform molecular docking studies, making docking an increasingly important tool in pharmaceutical research.

The molecular docking approach is useful to model the binding between a small molecule and a protein at the atomic level, allowing the characterization of the behavior of small molecules in the interacting domain of target proteins as well as elucidating fundamental biochemical processes [84].

The docking process involves two important steps:

- The prediction of the ligand conformation and *pose* referring to its position and orientation within these sites.
- The assessment of the binding affinity.

These two steps are related to sampling methods and their respective scoring schemes.

The docking efficiency depend on knowing the location of the binding site before docking processes. Information about the sites could be obtained by comparing the target protein in a family of proteins sharing a similar function or with co-crystallized proteins with other ligands. In case of blind docking, meaning the absence of knowledge about the binding sites, predicting cavity detection programs or online servers can be helpful to identify putative active sites within proteins.

The first elucidation for the ligand-receptor binding mechanism is the “lock-and-key” theory consisting on the ligand fitting into the receptor like lock and key. Later, the “induced-fit” theory was developed but improving the lock-and-key theory, stating that the active site of the protein is continually modified by interactions with the ligands as the ligands interact with the protein. The importance of this theory is that the ligand and receptor should be considered flexible during docking. Taking into consideration the limitation of computer resources, the most popular method of docking is performed with a flexible ligand and a rigid receptor. Recently many efforts have been made to solve the problem of the flexibility of the receptor, however, flexible receptor docking presents the major challenge for available docking methods [82].

1.6.2 APPLICATION EXAMPLES OF MOLECULAR DOCKING FOR DRUG DISCOVERY

Combining docking with other computational techniques and experimental data could be involved in analyzing drug metabolism to find useful information for successful applications of docking.

- DNA gyrase is a bacterial enzyme that affects negatively the supercoils into bacterial DNA and unwinds of DNA, so it is studied as antibacterial target. After HTS failure to find novel inhibitors of DNA gyrase, *de novo* design was used for this enzyme and succeeded to obtain several new inhibitors [85]. Firstly, 3D complex structures of DNA gyrase were analyzed with known inhibitors analyzed to get a common binding pattern. Searching the Available Chemicals Directory and a part of the Roche compound inventory, about 600 compounds were collected. Close analogs of these compounds were also considered, obtaining a total of 3000 compounds to be tested using biased screening. With the knowledge of 3D structures of the binding site a series of highly potent DNA gyrase inhibitors was generated.

- Another example is the validation of docking and scoring applied in cytochromes P450 and some heme-containing proteins. Docking against heme-containing complexes was difficult because certain ligands interact directly to the heme iron atom which requires a precise energetics of this contact for different chelating groups to be properly balanced with other energetic terms. In the case of the P450s, the environment above the heme group is very hydrophobic compared to other enzymes and some docking methods perform poorly on interactions driven entirely by lipophilic contacts [86].

- Concerning VS and HTS, comparative research has been applied to screen the inhibitors of the protein tyrosine phosphatase-1B (PTP-1B). A library of approximately 400,000 compounds was applied for HTS. Some compounds were found with IC50 values below 100 μM, corresponding. The most active had an IC50 value of 4.2 μM. 235,000 commercially available molecules were docked for VS into the crystal structure of PTP-1B (PDB code 1pty). After docking, the top 1000-scoring molecules were considered for

evaluation. In total 889 molecules were actually available, and after inspection 365 compounds were chosen for testing. As a result, 127 molecules were found to be active withIC50 <100 μM.

It should be noted is that the hits from VS and HTS are very different from each other and a combination of VS and HTS may be more helpful for lead discovery [87].

2. AIMS:

Cancer is a group of diseases affecting cell growth in an abnormal manner, with the potential to invade or spread to other parts of the body.

The inhibition of proteins that control multiple oncogenic pathways offers a solution to treat cancer. One protein involved in multiple cellular pathways is peptidylprolyl PIN1. PIN1, is an isomerase that is involved in many pathways of cancer.

Molecular docking, based on ligand-binding was used to design PIN 1 inhibitor. This inhibitor faces limits in cellular uptake which reduced its efficacy. This work aimed to increase the cellular uptake of the C17 drug through its crystallization by using a surfactant followed by surface coating with albumin. In order to investigate the antitumor effect of the drug, cell vialibility test was performed on different cancer lines.

3. MATERIALS AND METHODS

3.1. PIN 1 INHIBITOR DESIGN

To identify molecules that are capable to inhibit in vivo PIN1, our laboratory collaborator used consensus docking to model existing PIN1-ligand X-ray structures and screened a chemical database for candidate inhibitors. The resulting PIN1 inhibitor was named C17. The delivery of C17 is highly impeded by the solubility of the drug. The modifications with Pluronic acid, CtaBr and albumin coating are new systems to improve the PIN 1 inhibitor uptake by tumor cells.

3.2.NANOCRYSTAL PRODUCTION

3.2.1. PLURONIC ACID F-127

It is a type of poloxamer that is made with triblock copolymer the first one is a hydrophilic block poly(ethylene oxide) (PEO), the second is a hydrophobic block poly(propylene oxide) (PPO) and the third block is a repeated PEO as represented in the figure 11.

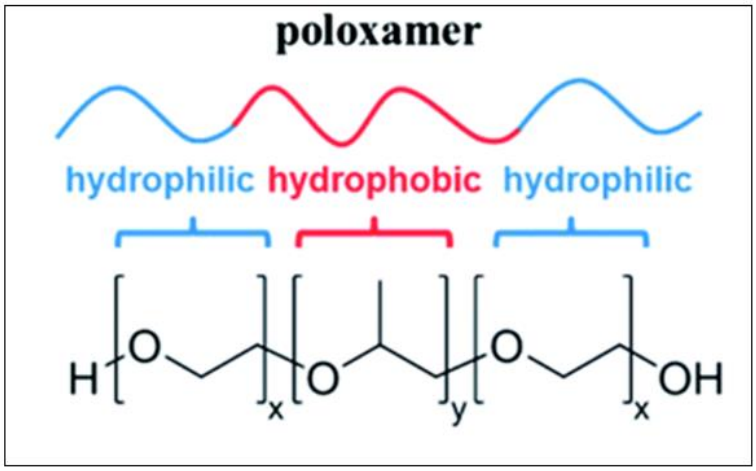

Figura 11: STRUCTURE OF PLURONIC ACID F-127

The F-127 has a molecular weight of 12600 g/mol and is composed by 65units of hydrophobic units (PO) together with 200 hydrophilic units (EO).

The utilization of the Pluronic acid in nanomedicine is related to their useful properties in solving drug delivery problems and from their intrinsic biological activity as well. In fact, F-127 is a potent biological response modifier capable to sensitize multidrug resistant (MDR) cancer cells and to enhance drug transport across cellular barriers through the inhibition of drug efflux transporters, such as P-glycoprotein and multidrug resistance proteins.

3.2.2. CtaBr

[(C16H33)N(CH3)3]Br, Cetyltrimethylammonium bromide is a quaternary ammonium surfactant. One of the main characteristics of CtaBr is his tendency, in dilute aqueous solutions, to self-assemble and form aggregates by exposing polar head groups to water while segregating hydrophobic tails from water. It has a molecular weight of 364.5g/mol and a C 1 Critical Micelle Concentration (CMC): 0.92 to 1.0 mM (water). Its aggregation number is 61 in water

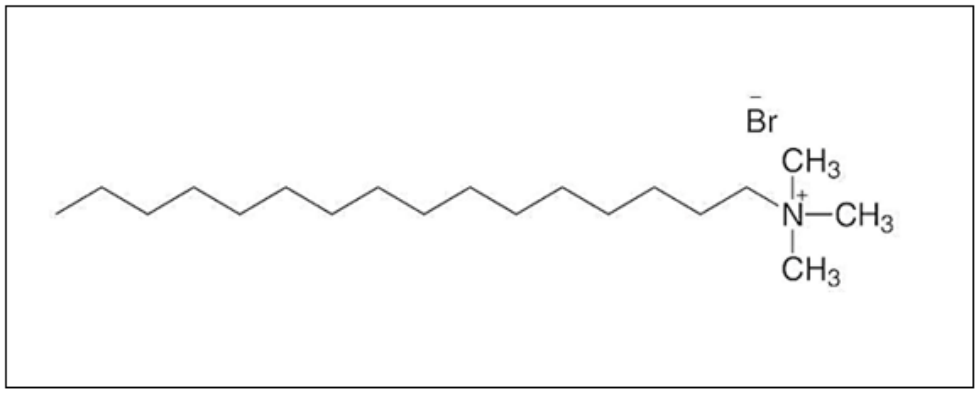

Figura 12: STRUCTURE OF CtaBr

3.2.3. NANOCRYSTAL SYNTHESIS

Although C17 has been successfully proven the potent inhibitor of PIN1 but one of major issue related with C17 is of its hydrophobic nature that preclude it for clinics. The delivery of the C17 was also another challenge ,as traditional drug delivery systems has been suffered with many issues, so there was an utmost need of a method that can carry maximum drug and can solve the solubility issue of C17. Therefore, very recently a study has been reported where they purposed the solubility and a career free delivery of paclitaxel (FDA approved drug) by crystallization method and finally the drug was stabilized by albumin nano crystals. Here we approached in a same way to achieve the nanocrystals of C17 and stabilized by albumin crystals.

In order to increase the loading efficiency of the C17, a crystallization followed by a coating method was assessed. The nano-crystallization was achieved by doing a mixture of C17 (6mg) and Pluronic acid (24mg) or CtaBr (2,4mg) both used as a surfactant. The mixture was fully dissolved in 2ml of chloroform in a rotavapor bottom flask, proceeding then with drying for 5 min. This process led to the formation of a thin monolayer on the flask of the bottom. In the next step 6 ml of MilliQ water were added at room temperature, then all the mixture was processed by bath sonication for 1min. The hydrated suspension obtained, were the hydrated suspension was probe-sonicated with a medium tip of 2.5 mm in an ice-water bath for 15 min with a power level of 40%.

The surface coating was performed by doing a mix of 1ml of C17-F (Pluronic acid) or C17-C (CtaBr) with 4mg of Albumin. The mixture was incubated and remained under rotation for 24 h at 30 RPM. The mixture was then centrifuged at 38000rpm at 4°C for 10min in order to remove the remaining surfactant and the unbounded albumin by discarding the supernatant. The obtained pellet was then resuspended inside 1ml of MilliQ water. Then was probe-sonicated with a medium tip of 2.5 mm in an ice-water bath for 15 min with a power level of 40% for 10 min. At this step a final centrifugation at 38000 RPM at 4°C for 10 min was performed, the obtained pellet was lyophilized.

3.3.NANOCRYSTAL CHARACTERIZATION

3.3.1. MORPHOLOGY ANALYSIS SAMPLE PREPARATION

The C17-F-Alb and C17-C-Alb were sonicated for 15 min in an ultra-sound bath. For each compound an amount of 25 ul was deposited on a copper grid (Holey film grid) containing 400 mesh. This process was followed by a staining process with Uranyl acetate (1%) for 2 min. The grid was then dried by using a paper filter before observation under microscope. Each compound has been observed by using a Transmission electron microscope FEI TECNAI G² at 100KV.

The images were obtained by using a digital camera VELETA (OLYMPUS SOFT IMAGING SYSTEM).

3.3.2. PARTICLE SIZE

The particles size was measured in sodium phosphate buffer (1mM, pH 7.4) with a zetasizer from Malvern Instrument.

3.4. CELL CULTURE

HCT116, OVCAR3, A2780 and SKOV3 cell line were purchased from the American Type Culture Collection (ATCC). Cells were maintained in their appropriate growth medium and plastic support at 37°C and 5% CO2.HCT116 colon cancer cells were grown in McCoy's 5A medium supplemented with10% FBS and antibiotics.

OVCA3, A2780 and SKOV3 OVARIAN cancer cells were grown in RPMI medium supplemented with 10% FBS and antibiotics.

Upon reached the confluence of 85-90%, the cells were detached by trypsin (Lonza). After trypsin inactivation, cells were resuspended in the appropriate medium and centrifuged 5 minutes at 1000 rpm. The resulting cellular pellet was resuspended using fresh medium and the cells counted by Bürker haemocytometer. The cells were seeded accordingly.

3.5. CELL VIABILITY

Cells (1000 x well) were seeded in 96 multiwell transparent microplate (Falcon) and treated with the appropriate compound concentrations (from 200mM to 0.01mM) after 24 hours.

After experimental timing (96 hours), cell viability was measured using the CellTiter-Glo® assay system (Promega) according to the manufacturer's instructions; a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present that indicate the metabolic activity of the cells.

Luminescence was assessed with microplate reader (Tecan M1000 plate reader).

The HCT116 colon cancer cell line and OVCAR3, SKOV3 and A2780 ovarian cancer cell lines were incubated with a range of concentrations of the drug from 200 μM to 0.01 μM in both free form and its encapsulated form with Pluronic acid only or pluronic acid and albumin. Cell viability was measured in an MTT-like assay after 96 hours of treatment with formulated C17 and free for form of C17. The results were analyzed using prism 6.

4. RESULTS AND DISCUSSION

4.1. C17 DRUG STRUCTURE

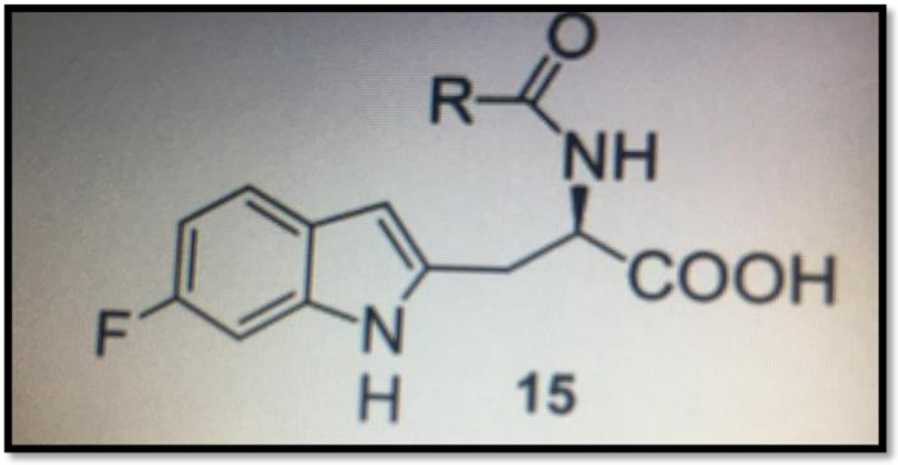

Figure 13 : C17 STRUCTURE

4.2. PREPARATION OF THE C17-F-ALB AND C17-C-ALB

4.2.1. THE C17-SURFACTANT MIXTURE

The nanoparticles were obtained from a crystallization step that involve the mixture of C17 and Pluronic acid F-127 (C17-F) or CtaBr (C17-C) as surfactant followed by hydration of the obtained dry film.

4.2.1.1. Z -average of the C17-C

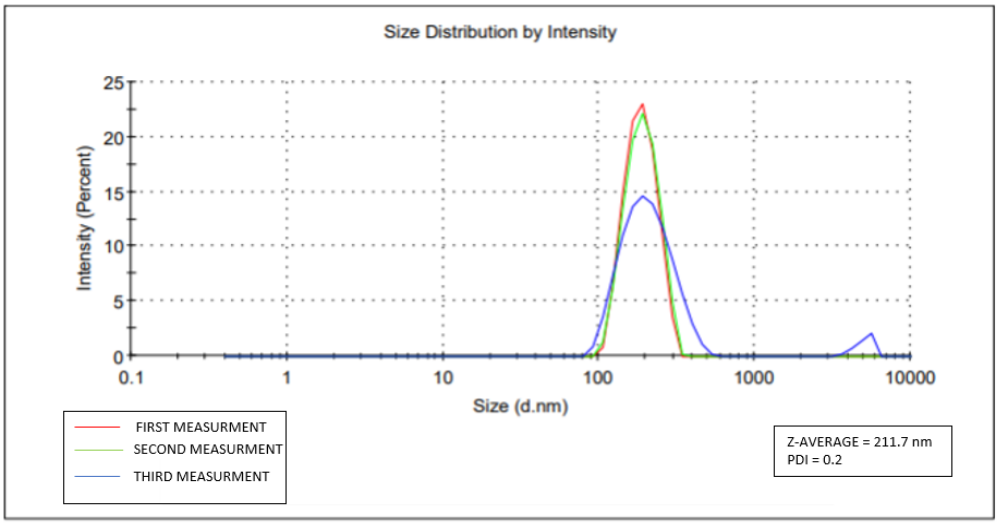

Figura 14: Z-AVERAGE AND POLYDISPERSITY MEASURMENT OF C17-C.

The mixture C17-C showed a z-average of 212 ± 77 nm. The polydispersity index used to estimate the average uniformity of a particle solution, for the compound C17 the value

corresponds to 0,26. According to the pharmaceutical range of PDI analysis, the C17-C particle solution can be considered monodispersed.

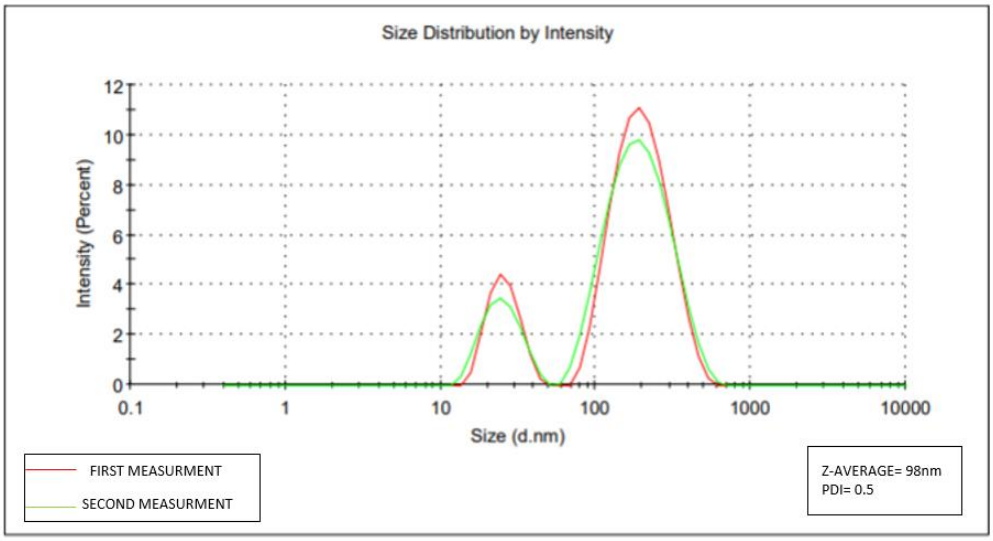

Figura 15: Z- AVERAGE AND POLIDISPERSITY OF THE C17- F

The C17-F showed a z-average of 98 ± 83,62 nm. In contrast to the C17-C, the obtained polydispersity index is estimate to 0,54 indicating the presence of some aggregation in the particle solution.

At the end, the relatively decrease in z-average is related to the fact that during the hydration the triblock polymer PEO -PPO-PEO start to dissolve and adsorb to the C17 particle via the hydrophobic PPO block and allow the hydrophilic PEO domains to be in contact with water. The F-127 play an important role in suppressing the crystal growth and their agglomeration.

4.2.2. THE ALBUMIN COATING OF THE C17-F

Albumin protein was added to the nanoparticle suspension and has been absorbed on their surface. The process involves a set of centrifugations to remove the excess of the albumin and the surfactant. This later excess removing allow to maintain the safety quality of the formulated drug.

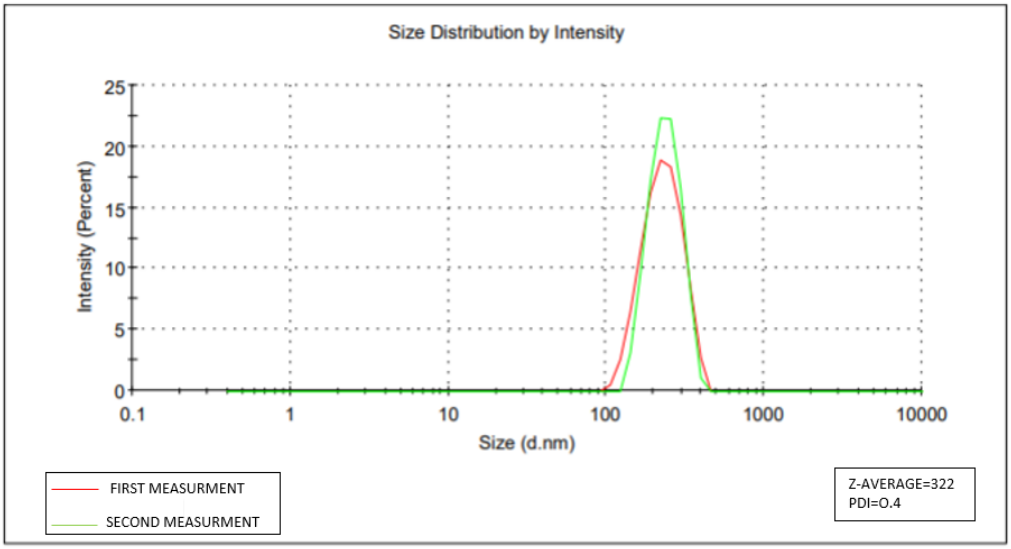

Figure 16: Z- AVERAGE AND POLYDISPERSITY MEASURMENT OF C17-F-Alb

The z-average of the albumin coated C17-F was 322± 64,71nm and the PDI obtained is 0.4. The obtained value of PDI confirm that albumin help in preventing agglomeration of the C17-F-Alb.

Further experiments are needed to adjust the correct value of the PDI and reduce the size of the obtained nanoparticles. Acting on the rate of hydration (from 1 min to 5s) it is possible to obtain zeta smaller and monodispersed nanoparticles.

4.3. THE Z-POTENTIAL ANALYSIS

Zeta potential measurements (based on the electrophoretic mobility of particles) are widely employed for the characterization of colloidal suspensions. It is also a technique used to evaluate protein absorption on bio-surfaces.

A low value of C17-F-Alb zeta potential measurement (-4,70 mV) was obtained. Thus, indicate an aggregation state of the nanoparticle solution. The negative zeta potential indicates the

presence of the Pluronic acid, that according to the structure it benefits to the colloidal stability in aqueous solution. Relatively, such low value can be related to the absorption of the albumin on the surface of the nanoparticles.

Further analysis must be performed at different level of preparation such as hydration or washing step in order to compare the different zeta potential value and to know how it is correlate to the adsorption process or binding processes.

4.4. THE MORPHOLOGY AND THE DIMENSION ANALYSIS:

TRANSMISSION ELECTRON MICROSCOPE MEASURMENT

4.4.1. TEM ANALYSIS OF THE C17-F

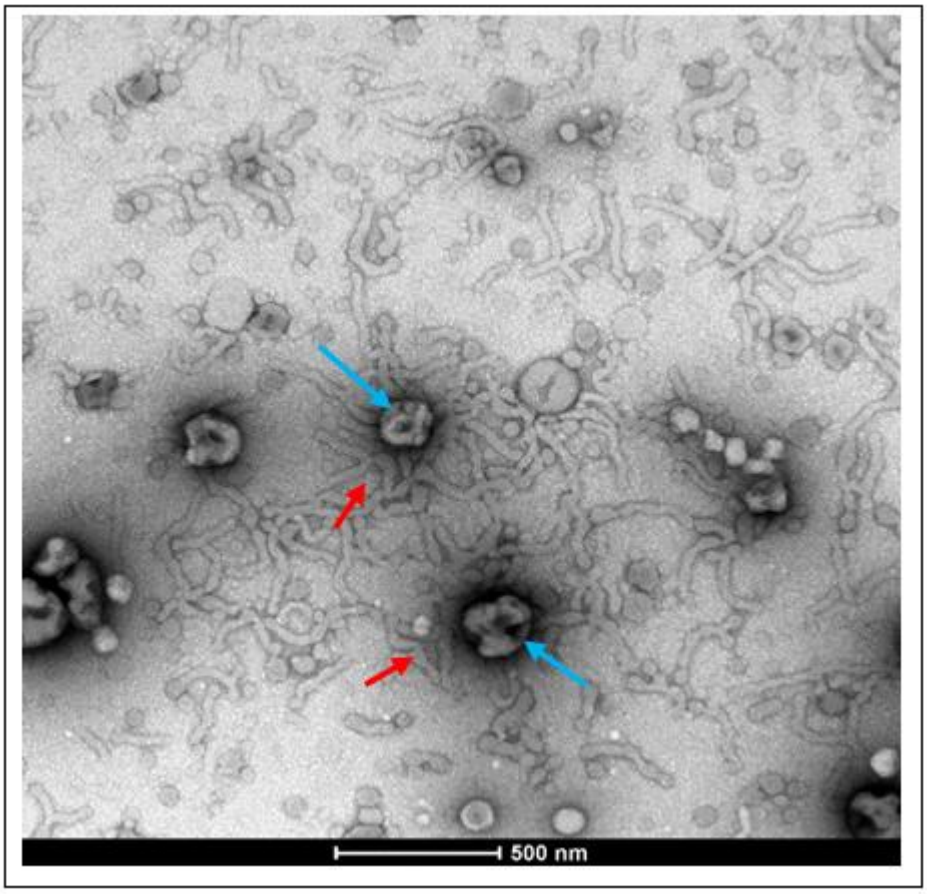

Figure 17: TEM IMAGE OF C17-F , The nanoparticles formulated with pluronic acid have a spherical shape (red arrow: C17 nanospheres ; blue arrow: micellar structure of F-127)

The image shows spherical nanoparticles, and the micellar aggregation structure of F-127 triblock polymer. The practical importance of such filamentous polymer micelles is to increase the circulating lifetime since they can be alligned with the blood flow.

4.4.2. COMPARATIVE TEM ANALYSIS OF C17-C-Alb AND C17-F-Alb

4.4.2.1. PARTICLE SIZE DISTRIBUTION

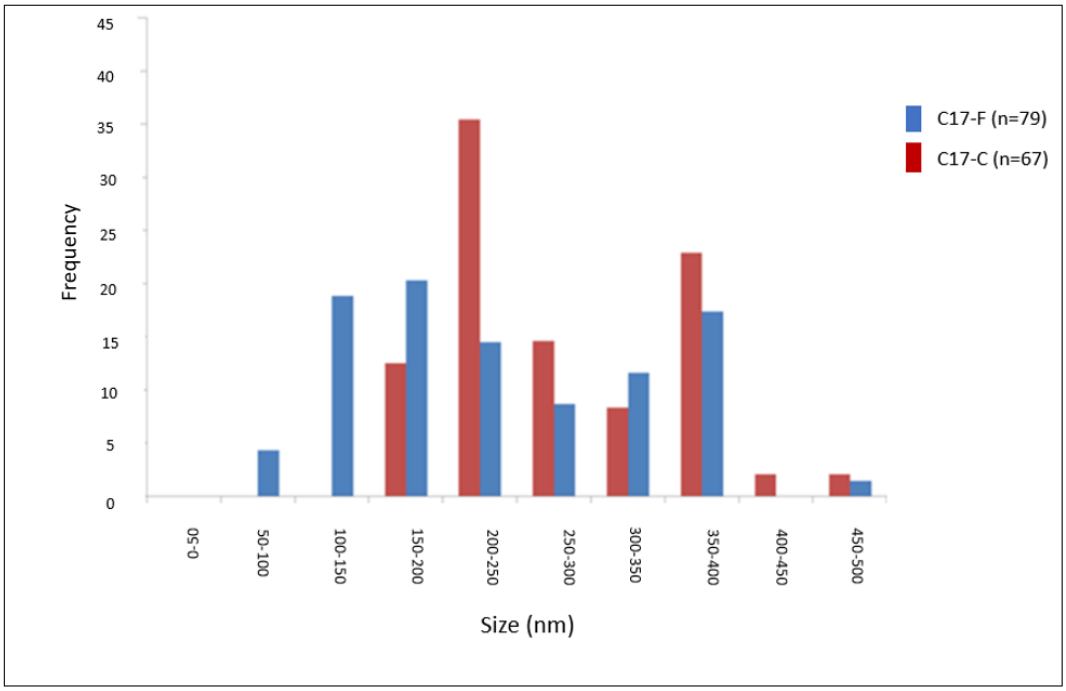

Figure 18: NANOPARTICLES SIZE DISTRIBUTION; This histogram represents the size distribution of C17-F (blue) and C17-C (red) both coated with albumin. (scale bar 0-600 nm)

The obtained nanoparticles formulated with Pluronic acid (*C17*-F) or with ctaBr (*C17*-C) coated with albumin showed an average diameter of 200 nm. This dimension is expected to be favorable for the uptake of the drug and to increase its circulation half-life

4.4.2.2. TEM ANALYSIS OF C17-C AND C17-F BOTH COATED WITH ALBUMIN

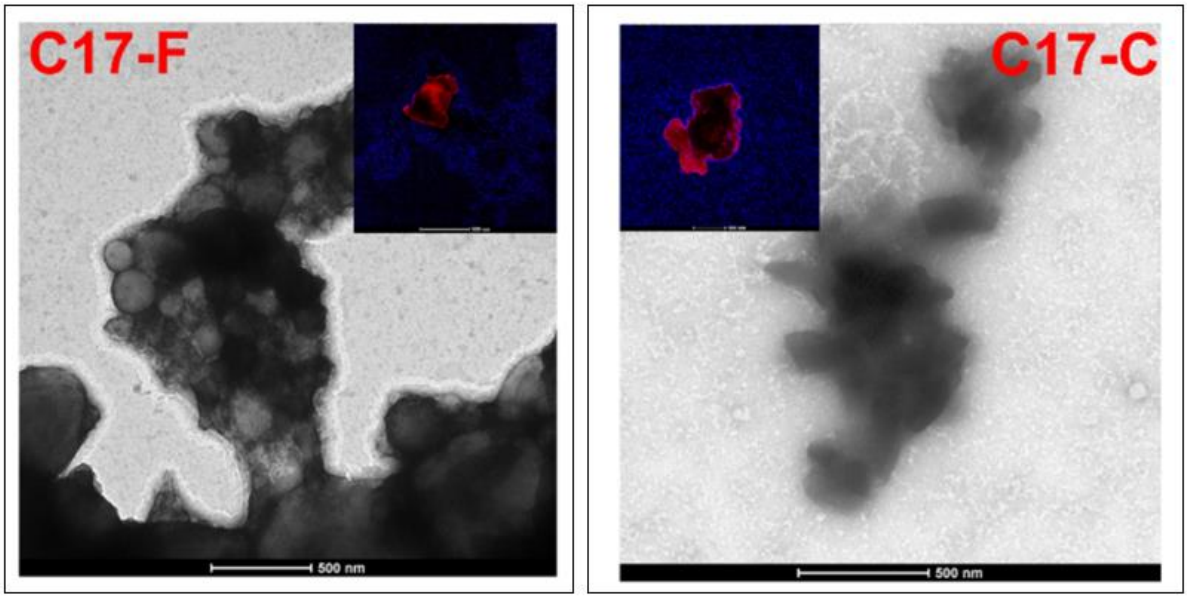

Figure 19: TEM IMAGES OF C17-C and C17-F COATED WITH ALBUMIN: THE IMAGE SHOW C17 WITH SURFACTANT (BLACK-RED) AND THE ALBUMIN (BLU)

The albumin adsorption on the nanoparticle is depicted in blue. In fact, albumin being the most abundant protein in plasma, involved in the trans-endothelial transport of nutrients and drugs, it binds to the nanoparticle surface and avoids the ER (endoplasmic reticulum) uptake by the cells.

According to these TEM analysis, the capability of albumin to adhere to the nanoparticles surface was higher when Pluronic acid was used as surfactant compared to CtaBr. In addition, the use of CtaBr as surfactant led to more agglomerates formation than the Pluronic acid. In consequence, we proceeded with the encapsulation of the C17 with albumin using Pluronic acid as surfactant.

4.5. EFFECT OF THE DRUG ON DIFFERENTS CELL LINES

4.5.1. COMPARATIVE EFFECT OF THE DIFFERENT DRUG FORMULATIONS

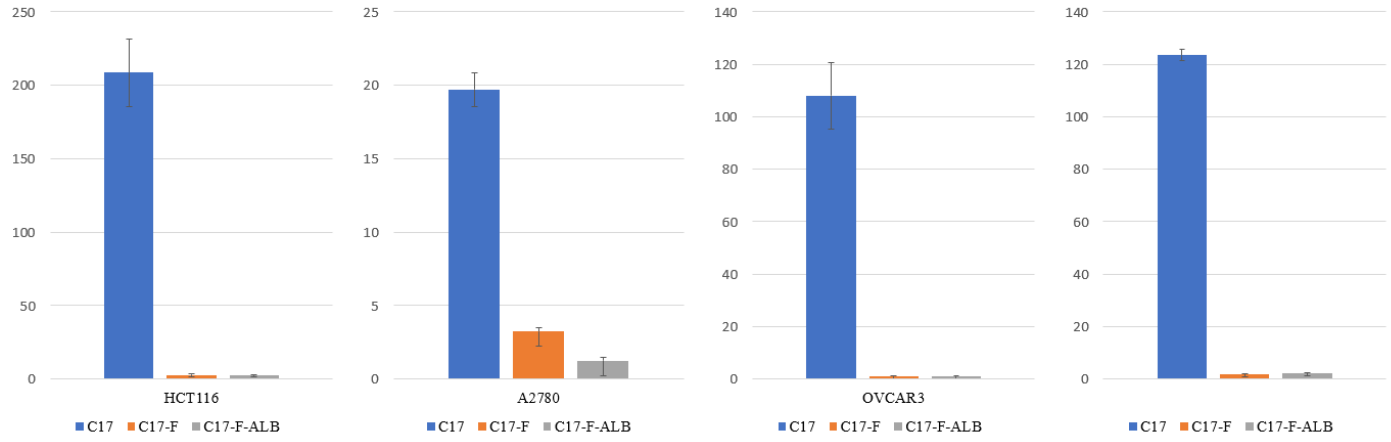

Figure 20: The reduction in IC 50 value was evident after the treatment of HCT116, A2780, OVCAR3 and SKOV3 cell lines with C17-Pluronic acid and C17-Pluronic acid coated with albumin compared to free C17. The Y axis represent the IC50 values and the X axis represents the different C17 drug formulations.

4.5.2. EFFECTIVE IC 50 VALUE

Cancer cell line	Tissue of origin	IC50 values (μM)		
		C17	C17-F	C17F/ALB
HCT116	Colon	208.5±22.9	2.615±0.5	2.338±0.4
OVCAR3	Ovary	108±12.7	0.34295±0.03	0.3701±0.01
SKOV3	Ovary	123.5±2.1	1.8805±0.04	2.215±0.2
A2780	Ovary	19.7±1.1	3.23±0.2	1.22795±0.2

Table 2: IC 50 VALUE OF C17, C17-F AND C17-F-Alb

The free form of C17, C17 modified with pluronic acid alone or with pluronic acid and albumin were tested in *in vitro* experiments to evaluate their antiproliferative potencies on cancer cells.

To this aim, four tumor cell lines were chosen, the human colorectal carcinoma HCT116, the human ovarian carcinoma OVCAR3 and A2780, and the human ovarian adenocarcinoma SKOV3. Overall, modified C17 produced a significant inhibition of cell viability with IC50 values sharply decreasing in all cell lines, reported in table 2 compared to the free form of C17 (Figure 20).

4.5.3. HCT 116 AND A2780 CELL NUMBER REDUCTION AFTER TREATMENT TREATMENT WITH C17-F-Alb

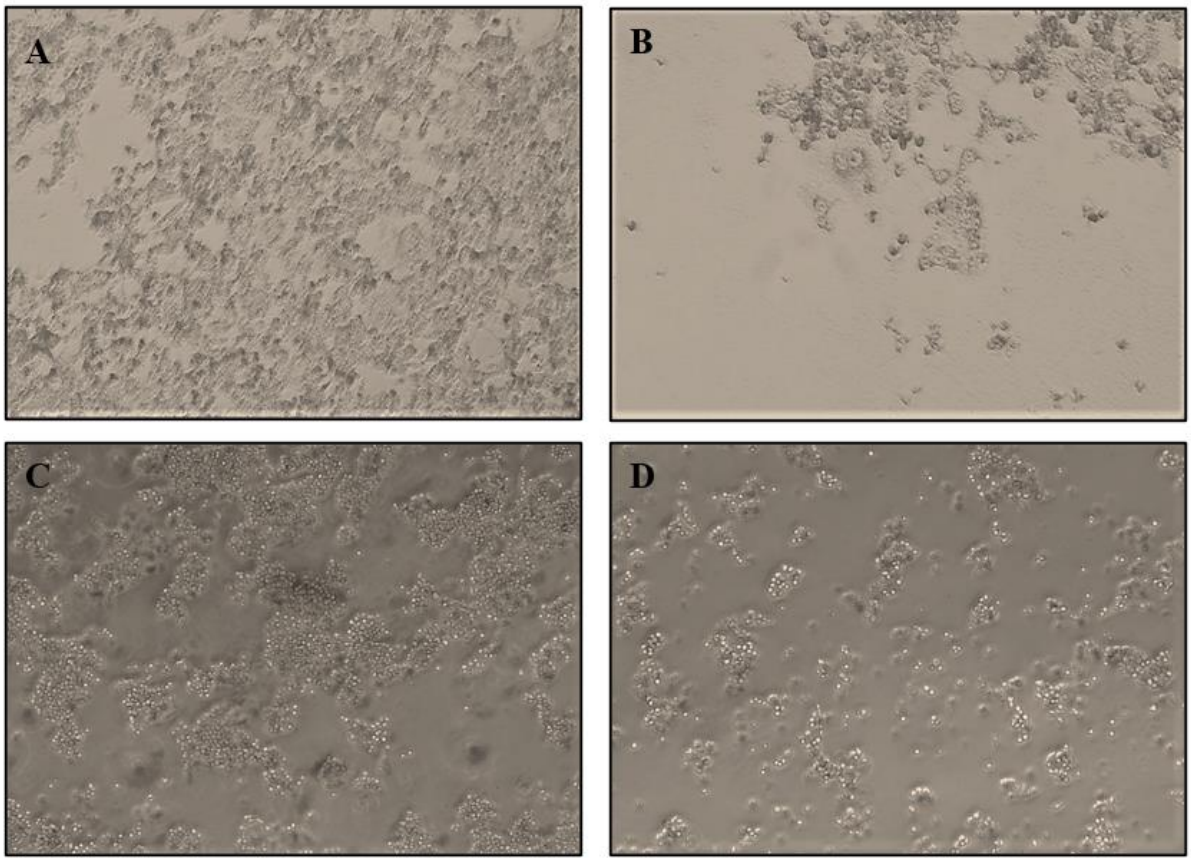

Figura 22: HCT116 and A2780 cells at 96 hours post treatment with C17-Pluronic acid coated with albumin.

A. HCT 116 colon cancer cells treated with 0.01 µM with C17-Pluronic acid coated with albumin. B. HCT 116 colon cancer cells treated with 200 µM with C17-Pluronic acid coated with albumin. C. A2780 ovarian cancer cells treated with 0.01 µM with C17-Pluronic acid coated with albumin. D. A2780 ovarian cancer cells treated with 200 µM with C17-Pluronic acid coated with albumin, the cells were examined with a NIKON ECLIPSE Ti Fluorescence microscope 4X

5. CONCLUSION AND FUTURE PERSPECTIVES

The therapeutic procedure regarding the treatment of cancer have demonstrated a certain grade of efficacy, however the application was limited due to the obstacles in drug delivery systems that target specifically the tumor cells thus, the benefits in terms of survival are still limited. The necessity of better therapeutics has been addressed by multiple studies.

The delivery systems could be enhanced with the use of nanomaterials as pharmaceutical drug carriers which increases antitumor efficacy. Nanoparticles (NP) present an important drug delivery potential for the administration of therapeutic drugs, offering numerous advantages such as better efficiency and low toxicity.

In this work, a carrier free nanoparticle formulation was developed to face the efficiency in the circulation stability of the compound C17. A process including a crystalization with pluronic acid F127 and an albumin coating allow the production of nanoparticles drugs that were able to decrease the IC50 value according to the drug alone when tested on different cancer lines.

This observation is due to the fact that the formulated drugs accumulated preferentially in cancer cell line respect to the free form of the C17.

In order to confirm the effectiveness of the drug further experiments need to be performed in order to evaluate the localization of the C17-F-Alb by doping it with a small amount of a fluorescent dye. In addition, other experiments should be performed to assess the molecular behavior of this compound in both normal and cancer cells.

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