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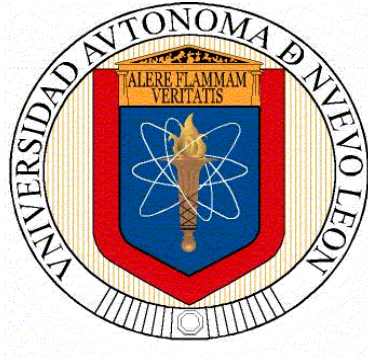

TESIS

STUDY OF THE ANTIMETASTATIC EFFECT OF PKHB1 IN BREAST, COLON, AND PANCREATIC CANCER CELLS

POR

RODOLFO MENDOZA REVELES

COMO REQUISITO PARA OBTENER EL GRADO DE MAESTRÍA EN CIENCIAS CON ORIENTACIÓN EN INMUNOBIOLOGÍA

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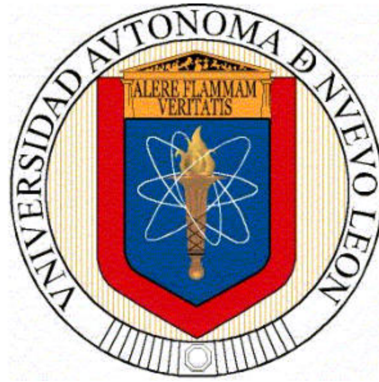

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By

RODOLFO MENDOZA REVELES

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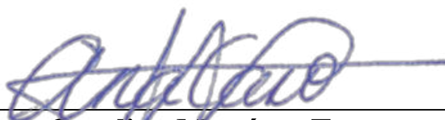

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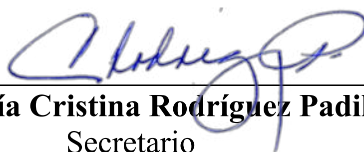

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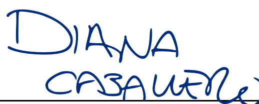

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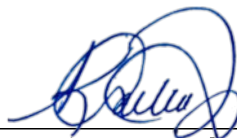
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Dra. Katiushka Arévalo Niño
Subdirectora de Posgrado

STUDY OF THE ANTIMETASTATIC EFFECT OF PKHB1 IN BREAST, COLON, AND PANCREATIC CANCER CELLS

Thesis Direction

Dra. Ana Carolina Martínez Torres
Director

Dr. Philippe Karoyan
Director Externo

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LIST OF ABBREVIATIONS

$\Delta\psi m$	Mitochondrial membrane potential
2APB	2-aminoethoxydiphenyl borate
ACD	Accidental cell death
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
Ann-V-APC	Annexin-V-allophycocyanin
CC20	Cytotoxic concentration 20
CC50	Cytotoxic concentration 50
CCSL	Sublethal concentration
CLL	Chronic lymphocytic leukemia
CSCs	Cancer stem cells
DAMPs	Damage-associated molecular patterns
Dant	Dantrolene
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum.
HE	Dihydroethidium
ICD	Immunogenic cell death
IP_3	Inositol 1,4,5-trisphosphate
IP_3Rs	Inositol-1,4,5-trisphophate receptors
PI	Propidium iodide
PIP2	Phosphatidylinositol 4, 5 bisphosphate
PLC	Phospholipase C
PMN	Pre-metastatic niche
QVD	Q-VD-OPH Inhibitor
RCD	Regulated cell death
ROS	Reactive Oxygen Species
RYRs	Ryanodine receptors
SPPS	Synthesized in solid phase.
TRP	Transient receptor potential
TSP-1	Thrombospondin-1
U73	U-73122 Inhibitor

ABSTRACT

Cancer is a broad group of diseases that are characterized by the uncontrolled growth of abnormal cells, which can invade surrounding tissues and/or spread to other organs (metastasis). Currently, metastasis is the cause of more than 90% of cancer deaths, among these, breast and colon cancer have the highest incidence of metastasis in women and men, respectively, while pancreatic cancer has one of the worst prognoses when metastasis is present, with 3% 5-year survival. In recent years it has been proposed to investigate new treatments that can inhibit this metastatic process; however, current metastasis inhibitors require being used in combination with cytotoxic therapies to eliminate the primary tumor, and most of these have significant side effects.

PKHB1, a thrombospondin 1 (TSP-1) derived peptide, was shown to induce cell death in distinct tumor and leukemic cell types via an unusual, caspase-independent, intra- and extracellular calcium-dependent pathway, without affecting non-tumor cells. Furthermore, it has been reported that TSP-1 can affect migration, invasion and proliferation of both healthy and cancer cells. Therefore, the objective of this thesis was to evaluate if PKHB1 has a cytotoxic and antimetastatic effect in metastatic breast, colon, and pancreatic cancer cells.

With the results of this thesis, we conclude that the mechanism of PKHB1-induced cell death in metastatic cancer cells has the same characteristics previously reported in leukemic and breast cancer cells. This mechanism is distinguished for being different from apoptosis and for inducing an increase in cytoplasmic Ca^{2+} via the PLC/IP_3R and store-operated Ca^{2+} entry pathways, two of the most important pathways in tumor cells, giving it a great advantage over other therapies and decreasing the possibility of generating drug resistance. Likewise, PKHB1 had an effect on the metastatic capacity of breast, colon, and pancreatic cancer cells, reducing their ability to replicate, migrate and invade other tissues. Therefore, we propose PKHB1 as a potential anti-metastatic treatment, even in highly metastatic cancers, which preserves its effect in different types of cancer.

RESUMEN

El cáncer es un amplio grupo de enfermedades caracterizadas por el crecimiento incontrolado de células anormales, que pueden invadir tejidos adyacentes y/o extenderse a otros órganos (metástasis). En la actualidad, la metástasis es la causa de más del 90% de las muertes por cáncer; entre ellos, el cáncer de mama y el de colon presentan la mayor incidencia de metástasis en mujeres y hombres, respectivamente, mientras que el cáncer de páncreas tiene uno de los peores pronósticos cuando hay metástasis, con un 3% de supervivencia a 5 años. En los últimos años se ha propuesto investigar nuevos tratamientos que puedan inhibir este proceso metastásico; sin embargo, los actuales inhibidores de la metástasis requieren ser utilizados en combinación con tratamientos citotóxicos para eliminar el tumor primario, y la mayoría de ellos tienen importantes efectos secundarios.

En los últimos años se ha demostrado que el PKHB1, un péptido derivado de la trombospondina-1 (TSP-1), induce muerte celular en distintos tipos de células tumorales y leucémicas mediante una vía de muerte celular atípica, independiente de las caspasas y dependiente del calcio intra y extracelular, sin afectar a las células no tumorales. Además, se ha informado de que la TSP-1 puede afectar a la migración, invasión y proliferación tanto de células sanas como tumorales. Por lo tanto, el objetivo de esta tesis fue evaluar si el PKHB1 tiene un efecto citotóxico y antimetastásico en células metastásicas de cáncer de mama, colon y páncreas.

Con esta tesis, concluimos que el mecanismo de muerte celular inducido por el PKHB1 sobre células cancerosas metastásicas tiene las mismas características previamente reportadas en células leucémicas y de cáncer de mama. Este mecanismo se distingue por ser diferente de la apoptosis y por inducir un aumento del Ca^{2+} citoplasmático a partir de las vías PLC/IP_3R y entrada de Ca^{2+} por salida de Ca^{2+}, dos de las vías más importantes en las células tumorales, para llevar a cabo su efecto citotóxico, dándole una gran ventaja sobre otras terapias y disminuyendo la posibilidad de generar resistencia a los fármacos. Asimismo, el PKHB1 tuvo un efecto sobre la capacidad metastásica de las células de cáncer de mama, colon y páncreas, reduciendo su capacidad para replicarse, migrar e invadir otros tejidos. Por lo que proponemos al PKHB1 como un potencial tratamiento anti-metastásico, aun en tipos de cáncer altamente metastásicos, el cual conserva su efecto en diferentes tipos de cáncer

.INTRODUCTION

Cancer is one of the diseases with the highest morbidity and mortality rates around the world, with an estimated 10 million deaths in 2020 (WHO, 2022).Resistance to treatments (mainly those that induce cell death by apoptotic pathways), and metastasis are the main causes of mortality, causing more than 90% of cancer-related deaths (Riggio et al, 2021). In the last decade, the use of new drugs that inhibit metastatic cancer cells have been proposed in order to reduce the number of deaths caused by this disease worldwide.

However, these therapies have major limitations, since they have a high rate of treatment-resistance due to the fact that their target proteins are not necessary for the survival of the tumor cell. They also present a lack of specificity towards the tumor cells, which causes side effects, and they need to be combined with other cytotoxic treatments to eliminate cancer cells at the primary tumor location.

This is why the search for new treatments that can inhibit the metastatic capacity of tumor cells by using Ca^{2+} signaling pathways has been proposed. This is because tumor cells, and especially cells with metastatic capacity, have a high rate of calcium dysregulations, since this ion is highly involved in survival, proliferation, cancer stem cell generation, epithelial-mesenchymal transition, migration, and invasion signaling pathways. However, these inhibitors depend on their combination with other cytotoxic treatments, so it is interesting to look for new treatments that can induce both effects.

In this regard, PKHB1, a thrombospondin-1 (TSP-1)-derived peptide, is of great interest, since it induces non-apoptotic cell death in tumor cells without harming non-tumor cells (Martínez-Torres et al, 2015, Uscanga-Palomeque et al, 2019). This type of cell death is characterized by using Ca^{2+} ion as a second messenger through the PLC-γ/IP_3 pathway, causing the activation of IP_3R and RyR channels, as well as the entry of extracellular Ca^{2+}. This mechanism gives this treatment an advantage over other treatments, due to the fact that this pathway is upregulated in most cancer cells. In addition, TSP-1 has been reported to have anti-metastatic effects by inhibiting the proliferation of healthy endothelial cells as well as skin carcinoma cells and metastatic cells that are in dormancy. Also, TSP-1

inhibits invasion and migration of oral squamous cell carcinoma cells and healthy endothelial cells.

However, it is unknown whether PKHB1 maintains any of the anti-metastatic effects reported in TSP-1. This is why we proposed to analyze the cytotoxic effect of this peptide on MDA-MB-231, HCT-116 and PANC-1 cell lines, which are derived of aggressive types of cancer, and its effect on their migration, invasion, and proliferation capacity.

BACKGROUND

1. Cancer

Cancer is described as a broad group of diseases that are characterized by the uncontrolled growth of abnormal cells, which have the capacity to invade surrounding tissues and/or spread to other organs (metastasis) (WHO, 2020). This disease is considered a leading cause of illness and mortality in the world, surpassing deaths caused by coronary heart disease or cerebrovascular disease, with an estimated 10 million deaths worldwide in 2020 (WHO, 2022).

Cancer development arises from the accumulation of diverse genetic mutations in the cells of an organism, which emulate Darwinian evolution, as each genetic change confers an advantage to increase their survival and reproduction, thus leading to a progressive transformation from normal cells to cancer cells.

These changes are known as the hallmarks of cancer (Fig. 1) and are defined as ten biological capabilities acquired in tumor development, which are used as a basis for encompassing all the complex aspects of this disease and organizing it in a rational way (Hanahan and Weinberg, 2011).

Figure 1. Hallmarks of cancer. In the center of the figure is the tumor microenvironment, a site composed of cancer cells, as well as cells of the immune system, fibroblasts and others that aid in tumor establishment. Listed around the microenvironment are the ten characteristics of a cancer cell accompanied by a symbol representative of each characteristic (Hanahan and Weinberg, 2011).

2. Hallmarks of cancer

Among the different characteristics of tumor cells, resistance to death and the capacity for invasion and metastasis stand out as the main causes of death in patients nowadays.

2.1. Resistance to cell death

Cell death is defined as the non-reversible degeneration of a cell's vital functions, resulting in the loss of cellular integrity (Galluzzi et al, 2018). It is now known that cell death can occur accidentally (ACD) or regulated (RCD) depending on the stimulus by which it occurs.

In the case of RCD, it is known that the cell can carry out signaling of its death by various pathways involving second messengers, such as Ca^{2+} ion and reactive oxygen species (ROS), and effector proteins or complexes, such as activation of caspases. This results in different cell death mechanisms that are carried out depending on the state of the cell, the ligand used and the receptor that initiates the signaling. Among the different types of RCDs described, apoptosis stands out for its high relevance in body homeostasis.

During tumor development, cancer cells undergo various physiological stresses, such as high levels of cell signaling produced by the expression of oncogenes and the generation of DNA damage associated with hyperproliferation, which causes the induction of the main cell death pathways associated with the body's homeostasis, thus one of the main characteristics of the cells of an already formed tumor is their ability to generate resistance to cell death. In recent decades it has been shown that cancer cells develop several strategies to restrict or prevent apoptosis (Fig. 2), including: loss of TP53 function, increased anti-apoptotic regulators (Bcl-2, Bcl-xL) or survival signals (Igf1/2) expression through downregulation of pro-apoptotic factors (Bax, Bim, Puma).

In addition, it has been described that tumor cells can overexpress proteins involved in calcium signaling pathways, such as proteins belonging to the phospholipase C (PLC), inositol triphosphate receptor (IP_3R), ryanodine receptor (RyR) and transient receptor potential (TRP) families. This is due to the fact that Ca^{2+} also has a prosurvival effect depending on the signaling pathway used (Prevarskaya et al, 2011). That is the case of

Ca^{2+} entry by an interaction between Orai1/Orai3, which leads to Ca^{2+} entry by oscillations that promote tumor cell proliferation (Bruce & James, 2020).

Figure 2. Mutations in the apoptotic pathway in tumor cells. The diagram shows some of the pathways by which both intrinsic and extrinsic apoptotic cell death is modulated, with the components that promote apoptosis in green and those that inhibit it in red. Likewise, components that are mutated or abnormally expressed in most tumors are denoted with an asterisk. (Johnstone *et al*, 2002).

2.2. Invasion and metastasis

Metastasis is defined as the development of secondary tumors in a distal tissue from the original tumor site. Due to the complexity of this process and the various steps involved, metastasis is considered the principal reason of cancer treatment failure, being the cause of death for more than 90% of cancer patients (Riggio et al, 2021).

Among the several types of cancer, colon and breast cancer have the highest percentage of metastasis in men and women respectively worldwide (Riihimäki et al, 2018), while pancreatic cancer is among the deadliest with a 5-year survival rate of 3% when metastasis is present (American Cancer Society, 2023).

In the last few years, it has been described that tumor cells do not passively migrate to a metastatic site, but first there is a remodeling of the potential metastatic site from the arrival of proteins and exosomes released by the main tumor (Peinado et al, 2017). This

step is referred to as premetastatic niche (PMN) formation and is of great importance in cancer treatment, since in this process a remodeling of the extracellular matrix and immunosuppression are induced, which favors tumor growth and evasion of the immune system even before there are even tumor cells at this site (Fares et al, 2020).

Tumoral cells migration into the blood vessels can originate from cancer stem cells (CSCs) or cancer cells that underwent an epithelial-mesenchymal transition (EMT). The first subtype is defined as a low population of cancer cells with characteristics similar to healthy stem cells, which can replicate in several tissues due to their low differentiation, being capable of self-renewal and generating tumor cells differentiated towards a specific tissue type (Tanei et al, 2009). Likewise, these CSCs present high heterogeneity, where the increase of proteins associated with treatment resistance stands out, as well as a low replication rate (Ding and Xie, 2018).

On the other hand, EMT is the transdifferentiation process by which tumor cells develop the ability to invade, resist stress, and spread. This is because cancer cells lose the need to interact with other cells and decrease their replication rate to a quiescent state. In addition to these characteristics, it has been shown that tumor cells can travel in the bloodstream in clusters of cells, providing them with a greater metastatic capacity as there are cells with different degrees of EMT and, therefore, giving them great heterogeneity (Fares et al, 2020).

In both cases, those cells that present this metastatic capacity have been characterized by deregulations in Ca^{2+} signaling pathways, which translates into a better capacity to cope with the different points of the metastatic process (Hammad & Machaca, 2021). In this sense, it has been reported that EGF induces alterations in IP_3R and RyR transcription levels, which cause EMT in the cell (Davis et al, 2013). On the other hand, RyR1 and IP_3R activation have been shown to be linked to CSC formation in melanoma and breast cancer models.

Furthermore, the interaction of STIM1 with Orai1 can control the generation of metastasis, due to its ability to modulate the activation of metalloproteases in the cell membrane and its capacity to mediate actomyosin assembly and the formation of focal adhesions. Finally,

a relationship has been seen between the overexpression of different PLC subtypes and the generation of metastasis in vivo in breast cancer models.

2. Standard treatments

There are many types of cancer treatment, which are administered according to the type and stage of the patient's cancer. These different treatments are due to the different histopathologies and the high heterogeneity of the different types of cancer depending on the tissue from which they originate and how advanced the tumor growth is. This causes each type of cancer to present variations in their sensitivity to current treatments and gives them a great capacity to generate drug resistance. For these reasons, different types of treatments have been created to combat this malignancy from different points of view.

The following is a description of the standard treatments reported by the WHO (WHO, 2022):

2.1. Surgery

This practice is based on a physical intervention in the tumor in order to remove the cancerous cells, as well as a small amount of surrounding normal tissue.

In the case of metastasis, surgery can only help to slow the spread of the cancer or eliminate certain side effects such as:

- The formation of an open wound in the breast (or chest) from the tumor.
- Spinal cord pressure due to a metastatic area.
- Liver blockage due to a metastatic area.

2.2. Radiotherapy

Radiation therapy is a treatment with high-energy rays (or particles) that destroy cancer cells. Radiation is usually used in combination with other treatments (especially surgery).

Side effects of radiation therapy include:

- Infection
- Weakness and fracture of the ribs.
- Damage to nerves in the arm.
- Development of another type of cancer called angiosarcoma.

2.3. Chemotherapy

These types of therapies use cytotoxic chemical compounds that eliminate cells with a high rate of division. Each of these therapies show differences in their chemical composition, the way they are administered, the treatment scheme, their effectiveness against a specific type of cancer and the side effects they cause in patients.

The efficacy with which treatment with these chemotherapies is carried out depends on several factors, such as: tumor size, level of tumor dissemination and molecular subtype to be treated.

These drugs enter the bloodstream and reach almost all areas of the body, which makes them useful in patients who present metastasis. However, the side effects they produce in the patient can weaken the immune system and the patient in general, as well as being lethal in very high doses. Therefore, their use must be careful when calculating the doses used.

Likewise, tumor cells show a great capacity to generate resistance to the type of cell death induced by most chemotherapies (apoptosis), which translates into resistance to treatment, leading to the use of more than one chemotherapy at a time.

2.4. Targeted therapies

As researchers learn more about the changes present in cancer cells, new types of drugs have been developed that specifically target these changes.

These drugs are systemic like chemotherapies, so they are useful in patients who present metastasis. In addition, these targeted drugs may work against some types of cancer that show resistance to chemotherapies.

It should be noted that, although these targeted treatments have helped to steadily reduce cancer mortality over the past three decades, they are usually not effective enough for implementation as individualized therapy (American Cancer Society, 2023). In addition, cancer cells show a high mutation rate, which causes heterogeneity at the same tumor site or metastatic point (Burrel et al, 2013), opening the possibility of generating resistance

against these treatments, since there is a great possibility of generating a population of cells that do not have the proteins targeted by this type of therapy.

3. New approaches to treatment

In the last two decades, two alternatives have been proposed to eliminate the problem of metastasis, the use of immunogenic cell death (ICD) inducers and the use of metastasis inhibitors.

3.1. Immunogenic cell death inductors

ICD is an immunostimulatory type of RCD, which has distinctive features that lead it to promote activation of the adaptive immune response specifically against endogenous antigens expressed by dying cells. These agents can stimulate the timely release of a number of soluble mediators called damage-associated molecular patterns (DAMPs) on a space-time defined basis (Kromer et al, 2013), which are recognized by both innate and adaptive immune system components, warning the body of a dangerous condition, leading to the activation of the immune response usually associated to the establishment of immunological memory (Wu and Waxman, 2018). Resulting in the body itself being the one that eliminates the tumor cells, managing to simultaneously remove those cells that are resistant to treatment.

However, in the clinical area, the theoretical benefits of the individual use of ICD-inducing therapies have not been observed. This is due to several factors among which the following stand out:

- The low specificity of most of these inducers, which causes damage to immune system cells and prevents their appropriate antitumor action.
- Evasion of the immune system by conglomerates of tumor cells circulating inactive in blood vessels. Which occurs by the interaction of these circulating tumor agglomerates with platelets, so that platelets encapsulate tumor cells so that they are not recognized (Pastushenko et al, 2018).
- The reduction of lymphatic vessels and suppression of T cells at metastatic sites, which is due to the action of exosomes released by the primary tumor (Fares et al, 2020).

3.2. Metastasis inhibitors

This is the most recent proposal in the quest to eliminate the problem of metastasis. In which it is proposed to inhibit the main steps involved in the generation of metastasis, being migration, invasion, evasion of the immune system in the bloodstream and replication in the metastatic sites the four most studied targets (Ganesh & Massagué, 2021).

This new line of research seeks for new treatments or previously approved treatments that can be reused to target the main proteins implicated in tumor cell migration and invasion (Pushpakom et al, 2019), having as targets, for example, extracellular matrix metalloproteinases, integrins such as αvβ3 and αvβ5, protein kinases of the SRC families, etc (Steeg, 2016) and, as reused treatments, calcium channel blockers, angiotensin II receptor inhibitors, β-blockers, thalidomide, etc (Dinić et al, 2020).

However, this approach has presented several limitations, among which according to Anderson et al (2019) are:

- The timing of the onset of the first phases of metastasis, since it has been reported that the first waves of cell migration can occur from very early stages of tumor generation. Therefore, when the patient is diagnosed, the first micrometastases are already present.

- The metastatic sites present molecular differences with the cells of the primary tumor. This means that treatments fail to inhibit metastasis of cells from metastatic sites.

- Lack of adequate models in clinical trials for new treatments. Because clinical studies are currently initiated in terminal phases of the patient, evaluating the capacity of the new treatment to eliminate the tumor, so that later, if it passes the test, it can be tested in earlier phases until it is tested as an adjuvant. However, this point of view does not consider treatments that do not focus on inducing cytotoxicity, eliminating them without proving their true purpose.

- The need to combine these treatments with cytotoxic therapies, since many anti-metastatic treatments do not induce a cytotoxic effect, and therefore do not eliminate the primary tumor.

- The lack of selectivity of these treatments, which causes the patient to show serious side effects. As in the case of beta-blockers, which cannot be used to attack metastasis due to the severity of their side effects.

Due to these, in recent years it has been proposed to attack the problem from another point of view, where new treatments are sought for their ability to use Ca^{2+} signaling pathways to achieve their effect. Considering that Ca^{2+} signaling has a critical role in the adaptive behavior of metastatic cells by modulating EMT, cancer stem cells and cell survival. (Iamshanova *et al*, 2017). Yet to date, there are no cancer treatments that are clinically approved, and very few undergoing clinical trials, that target the Ca^{2+} signaling machinery (Bruce & James, 2020).

These problems expose the need to search for new treatments that can induce regulated cell death in tumor cells, preferably with a selectivity towards these cancer cells, which can reduce or inhibit some of the main points of the metastasis process and use Ca^{2+} signaling pathways to achieve its effect. In this sense, PKHB1, a peptide derived from thrombospondin-1, seems to be of great relevance.

4. Thrombospondin-1

Thrombospondin-1 (TSP-1) belongs to a family of five multifunctional proteins that can be divided into two groups: a group of homotrimers with a procollagen-like region and repetitive regions (TSP 1 and 2) and a second group consisting of pure homopentamers (TSP 3, 4 and 5) (Adams & Lawler, 2011).

This protein controls cell fate by altering intracellular Ca^{2+} by binding to different receptors on the cell surface and to other components of the extracellular matrix. Its expression (in adult stages of organisms) is limited to the extracellular space of sites where there is tissue remodeling, where it determines the cellular phenotype, as well as the structure and composition of the extracellular matrix. In addition, TSP-1 and TSP-2 exhibit anti-angiogenic activities and are therefore capable of blocking tumor growth (Isenberg et al, 2009).

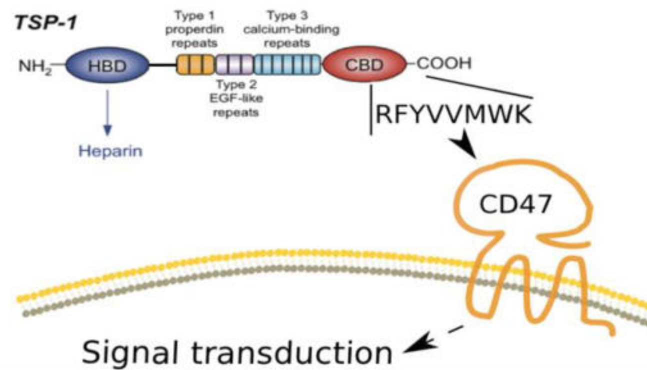

Figure 3. Thrombospondin-1. TSP-1 is made up of a globular N-terminal heparin domain (HBD), as well as three chains with type repeats: properdin (Type 1), epidermal growth factor (EGF, Type 2) and calcium binding (Type 3), linked by disulfide bridges, and another globular region at its C-terminal end (CBD) (Gene ID: 7057), which is the one that interacts with CD47. Adapted from Martínez-Torres, 2013.

Figure 3 shows the structure of TSP-1, as well as its interaction with CD47 (a transmembrane protein that is ubiquitously expressed in the body) via the VVM binding motifs found in the C-terminal domain of TSP-1. These domains are conserved among the five types of TSP, suggesting that CD47 binding may be a universal feature of this family of proteins.

These bindings are important since, specifically interactions with TSP-1, they have shown to produce intracellular signals that alter cellular Ca^{2+} and signaling by cyclic nucleotides, integrins and growth factors, controlling cell fate, viability, and stress resistance (Roberts et al, 2012).

4.1. Anti-metastatic effect of TSP-1

Currently, it is known that TSP-1 has diverse effects on the migration, invasion, and proliferation of different types of cells, both healthy and tumor cells, depending on the site with which TSP-1 interacts with the cell, the cell receptor used in this interaction and the situation in which the cell is found (Lawler, 2002). In this regard, it has been described that TSP-1 can inhibit endothelial cell migration and proliferation in several types of tumors, leading to angiogenesis inhibition and thus inhibiting tumor cell metastasis (Yee et al, 2009), likewise, TSP-1 overexpression has been shown to reduce invasiveness and

lymph node metastasis in oral squamous cell carcinoma cells(Yao *et al,* 2000). Similarly, TSP-1 prevents the growth of dormant cells in lung metastatic sites and its expression in skin carcinoma cells decreases the growth rate of these cells (Rofstad et al. 2004).

4.2. TSP-1 derived peptides

After the discovery that the VVM motif is responsible for CD47/TSP binding, several therapies such as the production of anti-CD47 antibodies and small peptides began to be developed. These peptides include 4N1 (1016-RFYVVMWK-1024), which is highly conserved throughout TSP species and isoforms (Kosfeld and Frazier, 1993) and from which the peptide 4N1K (K-RFYVVMWK-K) was developed, a 10 amino acid peptide corresponding to the sequence of 4N1 flanked by two lysine residues. This addition of the lysine residues was done to improve the solubility of 4N1 without affecting the binding to CD47 (Gao et al., 1996).

However, this improvement in the solubility of 4N1 was still not good enough to administer the treatment in a soluble form, limiting the potential of its therapeutic use, so a second peptide derived from 4N1 was synthesized which shares the same amino acid sequence as 4N1K, but changing the L-lysines at the ends for their enantiomers. This peptide was named PKHB1 and was the first peptide derived from TSP-1 with sufficient solubility to have a therapeutic use.

Nevertheless, PKHB1 still shows a tendency to aggregate and sediment, which is why two more modifications were made to the peptide in order to improve its solubility and reduce the interactions that occur between molecules of the same peptide. This new peptide was named PKT16 and is characterized by having the same sequence as PKHB1 with an N-methylation in the arginine residue and a norleucine instead of methionine (k(N-Me)RFYVV(Nle)WKk).

4.3. Induction of RCD by TSP-1-derived peptides

The biological consequences of the CD47/TSP-1 interaction is broad and depend on the type of cell, its association with other molecules, its conformation, its distribution on the cell surface, the mode of anchoring and the particular situation in which they occur

(Martínez-Torres, 2013). This is because the mechanisms activated by this interaction play a key role in the regulation of the organism's homeostasis.

Among these consequences, in 1999 it was observed that the activation of CD47 by molecules with the VVM motif (monoclonal antibodies, TSP-1 and 4N1K) induces a MCR in chronic lymphocytic leukemia (CLL) cells (Mateo et al, 1999). This finding led to research on the induction of MCR in various types of cancer (acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), breast cancer, among others).

With this, it could be observed that CD47-induced MCR in cancer cells conserves the following characteristics: they are fast processes, independent of caspases, present depolarization of the mitochondrial membrane without release of proapoptotic proteins, production of reactive oxygen species, exposure of phosphatidylserine, permeabilization of the plasma membrane and null DNA fragmentation or chromatin condensation (Martínez-Torres, 2013).

Additionally, PKHB1 is reported to induce immunogenic cell death in leukemic and breast cancer cells, through a cell death mechanism conserved in the different cells in which it was evaluated, this mechanism is characterized by being caspase-independent and inducing PLC activation that leads to inositol triphosphate (IP_3) production, that in turn activates IP_3R in the endoplasmic reticulum, which downstream promotes extracellular Ca^{2+} entry, activation of the RyR, mitochondrial damage, increased production of ROS and, ultimately, cell death (Martinez-Torres et al, 2015, Uscanga-Palomeque et al, 2019, Calvillo-Rodriguez et al, 2022).

This same cell death mechanism has been reported with PKT16 in CLL cells, so Pramil et al (2019) suggested that the peptide-induced pathway of cell death is unaffected upon changes in the backbone of each peptide. Furthermore, in this article, the induction of cell cycle arrest is added as one of the characteristics of the cell death pathway mechanism induced by TSP-1-derived peptides and CD47 agonists (Pramil et al, 2019). In this sense, considering the anti-metastatic effects of TSP-1, it is interesting to evaluate the effect of the peptides on tumor cells with high metastatic capacity, in order to see if they have an effect on the main metastatic processes. To this end, in this work we evaluated the

cytotoxic effect of PKHB1 on metastatic breast, colon and pancreatic cancer cells, and its effect on cell migration, invasion and proliferation capacity.

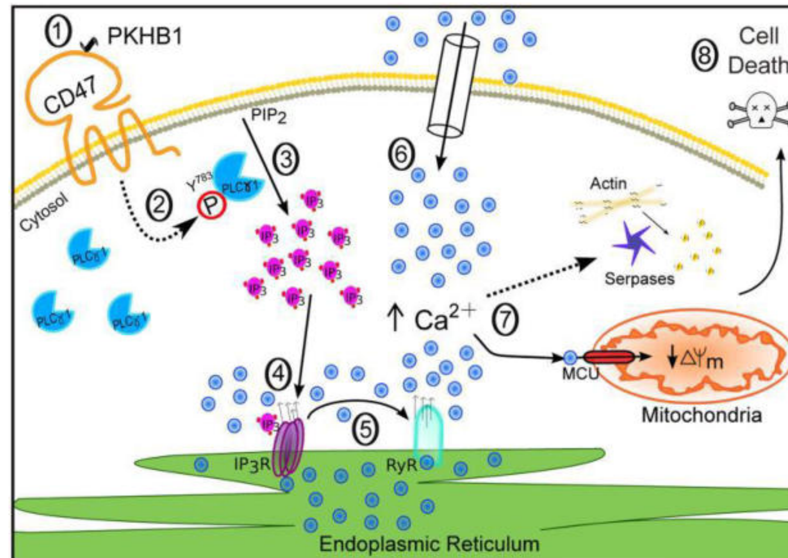

Figure 4. Schematic representation of PKHB1-induced MCR in cancer cells. PKHB1 interaction with CD47 (1) induces phosphorylation of PLC-γ (2). Subsequently, PLC-γ cleaves phosphatidylinositol 4, 5 bisphosphate (PIP2) producing inositol 1, 4, 5 triphosphate (IP_3) (3), which binds to the IP_3R receptor causing Ca^{2+} release (4). The increase in cytosolic Ca^{2+} activates RyR channels in the ER (5). ER Ca^{2+} depletion causes plasma membrane Ca^{2+} channels to open, resulting in massive Ca^{2+} entry into the cell (6). High cytoplasmic Ca^{2+} concentrations promote activation of serine proteases, actin depolarization, and mitochondrial damage (7), culminating in cell death (8). (Martinez-Torres et al, 2015).

JUSTIFICATION

Cancer represents the second most common cause of death from noncommunicable diseases around the world, with the generation of metastases being the main reason, as it causes more than 90% of these deaths. Despite the great efforts that have been made in the last decade, there is still no specific treatment to eliminate this problem, because the treatments focused on inhibiting metastatic processes have presented limitations such as the lack of an adequate preclinical model, the generation of resistance to these drugs, the need to be used in combinatorial treatments and the generation of side effects in patients due to the lack of specificity of the treatments. These lead to the continuous use of treatments focused only on inducing cytotoxicity of tumor cells, although this approach does not protect the patient from recurrence caused by the generation of metastases.

In this sense, it has been reported that TSP-1 has anti-metastatic effects, since it inhibits the proliferation of endothelial cells, skin carcinoma cells, metastatic cells that are in dormancy, and the migration and invasion of oral squamous cell carcinoma cells. Similarly, PKHB1, a peptide derived from TSP-1, has been shown to induce cell death in different types of cancer, without affecting healthy cells. The cell death induced by PKHB1 is characterized by being caspase-independent and Ca^{2+}-dependent, presenting an increase in intracellular Ca^{2+} due to activation of the PLC/IP_3 pathway and an influx of extracellular Ca^{2+}.

However, until this work, it was unknown whether PKHB1 induced cell death in colon and pancreatic cancer cells, nor the mechanisms involved. Thus, in this work we evaluated the cell death mechanism induced by PKHB1 on HCT-116 and PANC-1 lines. Additionally, it is also unknown whether PKHB1 presents the anti-metastatic effects reported in TSP-1. This is why in this thesis it was also evaluated the effect of PKHB1 on the migration, invasion, and proliferation capacity of the MDA-MB-231, HCT-116 and PANC-1 cell lines, since these are the most used models to evaluate the metastatic processes in breast, colon, and pancreatic cancer cells, respectively.

HYPOTHESIS

PKHB1 induces Ca^{2+}-dependent and caspase-independent cell death in breast, colon, and pancreatic cancer cells, also affecting their metastatic capacity.

GENERAL OBJECTIVE

To analyze the cytotoxic and antimetastatic effect of PKHB1 on MDA-MB-231, PANC-1 and HCT-116 lines.

SPECIFIC OBJECTIVES

1. To evaluate the PKHB1-induced cytotoxicity in MDA-MB-231, PANC-1 and HCT-116 lines.
2. To determine the molecular characteristics of PKHB1-induced cell death in MDA-MB-231, PANC-1 and HCT-116 lines.
3. To analyze the cell migration of MDA-MB-231, PANC-1 and HCT-116 lines after treatment with PKHB1
4. To evaluate the invasion ability of MDA-MB-231, PANC-1 and HCT-116 lines after treatment with PKHB1.
5. To determine the replicative capacity of MDA-MB-231, PANC-1 and HCT-116 lines after treatment with PKHB1.

MATERIALS AND METHODS

1. Cell culture

HCT 116 (ATCC CRL-247, human colon cancer cell line), MDA-MB-231 (ATCC HTB-26, human breast cancer cells), and PANC-1 (ATCC CRL- 1469, human pancreatic cancer cells) were obtained from the ATCC. Cells were maintained in DMEM-F12 medium supplemented with 10% FBS, 100 U/mL penicillin-streptomycin (GIBCO by Life Technologies, Grand Island, NY, USA), and incubated at 37°C in a controlled humidified atmosphere with 5% CO_2. Cell count was carried out using Trypan blue (0.4% Sigma-Aldrich), a Neubauer chamber and an optic microscope (Zeiss Primo Star) as proposed by the ATCC's standard protocols.

2. Peptides

Thrombospondin-1-derived peptide, PKHB1, was provided by Dr. Philippe Karoyan, who performs the development, synthesis, and production of the peptides. Briefly, the peptides are synthesized in solid phase (SPPS) on a Fmoc-Lys (Boc)-Wang resin, as previously described (Dènefle et al, 2016; Martinez-Torres et al, 2015).

3. Cell death analysis

HCT-116, MDA-MB.231, and PANC-1 cells were seeded (5×10^4 cells/well) in 24-wells dishes and left untreated or treated for 2 h with PKHB1 (as indicated, with 100, 150, 200, 250 and 300 μM) to obtain the sublethal concentration (CC_{SL}) and the cytotoxic concentrations that killed 20 or 50% of the cells (CC_{20} o CC_{50}). Then, annexin-V-allophycocyanin (Ann-V-APC 0.1μg/ml; BD Pharmingen, San Jose CA, USA), and propidium iodide (PI, 0.5μg/ml Sigma-Aldrich) were used to assess phosphatidylserine exposure, respectively, in a BD Accury C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (total population 10,000 cells). Data was analyzed using FlowJo software (LLC, Ashland, OR, USA).

For cell death inhibition assays, cells were pre-incubated for 20min at 37°C, with the extracellular Ca^{2+} chelator: BAPTA (2.5mM for MDA-MB-231 and 2mM for HCT-116 and PANC-1), the pan-caspase inhibitor: Q-VD-OPH (QVD, 10μM), the phospholipase C (PLC) inhibitor: U-73122 (U73, 1.5μM for MDA-MB-231 and 1μM for HCT-116 and PANC-1), the inositol triphosphate receptor (IP_3R) inhibitor: 2-aminoethoxydiphenyl

borate (2APB, 30uM) and the Ca^{2+}-dependent Ca^{2+} channel (RyR) inhibitor: Dantrolene (Dant, 40uM) (CalbioChem, Merck, Billerica MA, USA).

4. Intracellular Ca^{2+} levels assay

HCT-116, MDA-MB.231, and PANC-1 cells (5×10^4 cells/well) in 24-wells plates (Life Science) were pre-incubated or not with 2.5 mM BAPTA and then treated for 2 h with PKHB1 (CC_{50}) or left untreated in medium. Then, cells were detached, washed with RINGER buffer without Ca^{2+}, and resuspended in 200 µL of the same RINGER buffer with 0.001 µg/mL of Fluo-4 AM (Life Technologies) and 0.001 µg/mL of Pluronic F-127 (Life Technologies), incubated at 37°C for 30 min. Next, cells were washed with RINGER buffer w/o Ca^{2+} and assessed by BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (total population 10,000 cells). Data was analyzed using FlowJo software (LLC, Ashland, OR, USA).

5. Loss of mitochondrial membrane potential analysis

HCT-116, MDA-MB.231 and PANC-1 cells (5×10^4 cells/well) in 24 wells dishes (Life Science) were treated for 2 h with PKHB1 (CC_{50}) or left untreated in medium. Then, cells were detached, stained with 500 nM TMRE (Sigma-Aldrich) and incubated at 37°C for 30 min. Next, cells were washed with PBS and assessed by BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (total population 10,000 cells). Data was analyzed using FlowJo software (LLC, Ashland, OR, USA).

6. ROS generation analysis

HCT-116, MDA-MB.231 and PANC-1 cells (5×10^4 cells/well) in 24 wells dishes (Life Science) were treated for 2 h with PKHB1 (CC_{50}) or left untreated in medium. Then, cells were detached, stained with 2.5 µM dihydroethidium (HE) (Invitrogen), and incubated at 37°C for 30 min. Next, cells were washed with PBS and assessed by BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (total population 10,000 cells). Data was analyzed using FlowJo software (LLC, Ashland, OR, USA).

7. Scratch assay

HCT-116, MDA-MB.231 and PANC-1 cells (2×10^5 cells/well) in 24 wells plates (Life Science) were treated for 2 h with PKHB1 CC_{SL}, CC_{20}, and CC_{50} or left untreated in

medium. After this, a scratched area was created using a sterile 200μL pipette tip on an 100% confluence area. Then, the cells were washed with PBS, fresh medium was added, and the cells were incubated at 37°C. Cells migration into the wound surface were determined under microscope at 24, 48, and 72H. The ratio of cell migration was calculated with ImageJ as the percentage of the remaining cell-free area compared with the area of the initial scratched area by using a plugin developed and optimized by Suarez-Arellano et al (2020).

8. Transwell invasion assay

HCT-116, MDA-MB.231 and PANC-1 cells (5×10^4 cells/well) in 24 wells plates (Life Science) were treated for 2 h with PKHB1 CC_{SL}, CC_{20}, and CC_{50} or left untreated in medium. The upper compartment of Transwell (Corning) chamber was coated with 16 mg/ml cultrex BME (R&D Systems™). Then, cells were detached, and 4×10^4 living cells/well were seeded into the upper chamber of each Transwell with serum free medium, lower chambers contained fresh medium supplemented with 10% FBS. Cells were incubated at 37°C. After left 48 or 72H, the cells remaining at the upper surface of the membrane were removed using a swab, whereas those cells that had invaded to the lower membrane surface were fixed with 70% ethanol (-20°C) at 37°C (1h) and stained with crystal violet 0.5% at 37°C (2h). The membrane was photographed under microscope and the ratio of cell invasion was measured using ImageJ by converting the image to a 16-bit photograph and then using ImageJ's thresholding tool to remove background noise and finally extracting the percentage of area covered by the cells in the image. For this analysis, 4 pictures of each membrane were taken, and the average invasion rate was calculated.

9. Clonogenic assay

HCT-116, MDA-MB.231 and PANC-1 cells (1×10^5 cells/well) in 24 wells dishes (Life Science) were treated for 2 h with PKHB1 CC_{SL}, CC_{20}, and CC_{50} or left untreated in medium. After this, cells were detached, and 100 cells/well were seeded into 6 well dishes with DMEM 10% SFB. Cells were allowed to grow for 10 days at 37°C. Then, the cells

were fixed with 70% ethanol (-20°C) at 37°C (1h) and stained with crystal violet 0.5% at 37°C (2h). Colonies that were visible by eye were counted manually with ImageJ.

10. Statistical analysis

Data obtained *in vitro* with a normal distribution were analyzed by Student's t-test (parametric) for paired samples. GraphPad Prism 7 statistical software was used for this purpose. Experiments were repeated at least 3 times and values of $*p < 0.05$ were considered significant.

RESULTS

1. PKHB1 induces a concentration-dependent cytotoxic effect in metastatic breast, colon, and pancreatic cancer lines.

To evaluate the effect of PKHB1 on metastatic breast, colon, and pancreatic cancer cells, the first step was to determine whether it had a cytotoxic effect on the MDA-MB-231, HCT 116 and PANC-1 tumor lines. Figure 5 shows that the peptide induces a cytotoxic effect in the three cell lines, which increases proportionally to the peptide concentration used. Presenting a cytotoxic concentration (CC) that was sublethal (CC_{SL}) at 100μM in MDA-MB-231 (Figure 5A) and 150μM in HCT-116 and PANC-1 (Figures 5B and 5C, respectively), a CC of 20% at 150 μM in MDA-MB-231 (Figure 5A) and 200μM in HCT-116 and PANC-1 (Figures 5B and 5C, respectively), as well as a CC_{50} at 200μM in MDA-MB-231 (Figure 5A) and 250μM in HCT-116 and PANC-1 (Figures 5B and 5C, respectively). Finally, it was decided to use the concentrations of .

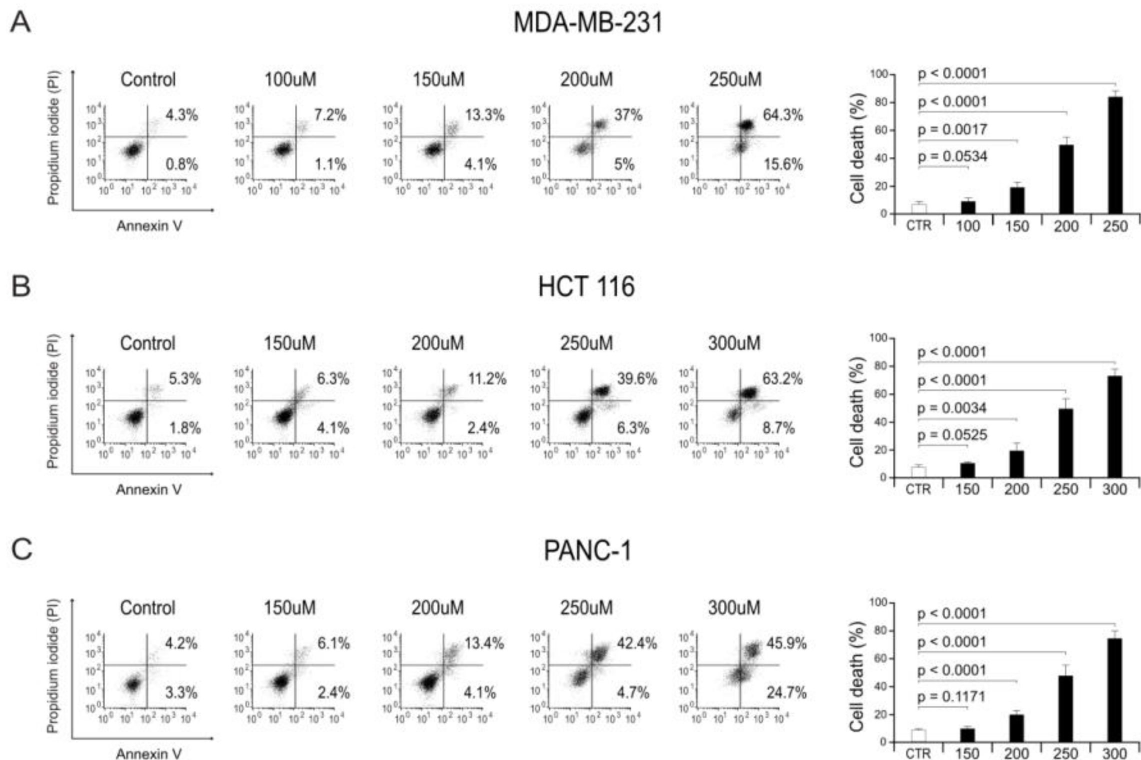

Figure 5. PKHB1 induces cell death in metastatic breast, colon, and pancreatic cancer lines. Cell death was determined by flow cytometry using Annexin V labeling bound to Allophycocyanin (Annexin V) and propidium iodide (PI). The dot plots (left) and their respective

graphs (right) of PKHB1-treated MDA-MB-231 (5A), HCT 116 (5B) and PANC-1 (5C) cells are shown in the figure. Untreated cells were used as controls. Graphs represent the average of at least three independent experiments performed in triplicate (± SD).

2. PKHB1-induced cell death is caspase-independent and Ca^{2+}-dependent.

Once the cytotoxic effect of PKHB1 was proven, it was decided to evaluate whether it induced the molecular characteristics of death previously observed in leukemic and breast cancer cells by Martínez-Torres et al (2015) and Calvillo-Rodriguez et al (2022). For this, cells were pre-incubated with BAPTA, QVD, U73122, 2APB and Dantrolene, evaluating whether the peptide maintained its cytotoxic effect in the presence of these inhibitors. Figure 6 shows MDA-MB-231 (Figure 6A), HCT 116 (Figure 6B) and PANC-1 (Figure 6C) cells treated with PKHB1, where it is observed that the inhibitor QVD had no effect on the induction of cell death produced by the peptide, whereas the extracellular Ca^{2+} chelator (BAPTA), the PLC inhibitor (U73) and the inhibitors of the IP_3R and RyR ER channels (2APB and DAN) were able to significantly reduce cell death.

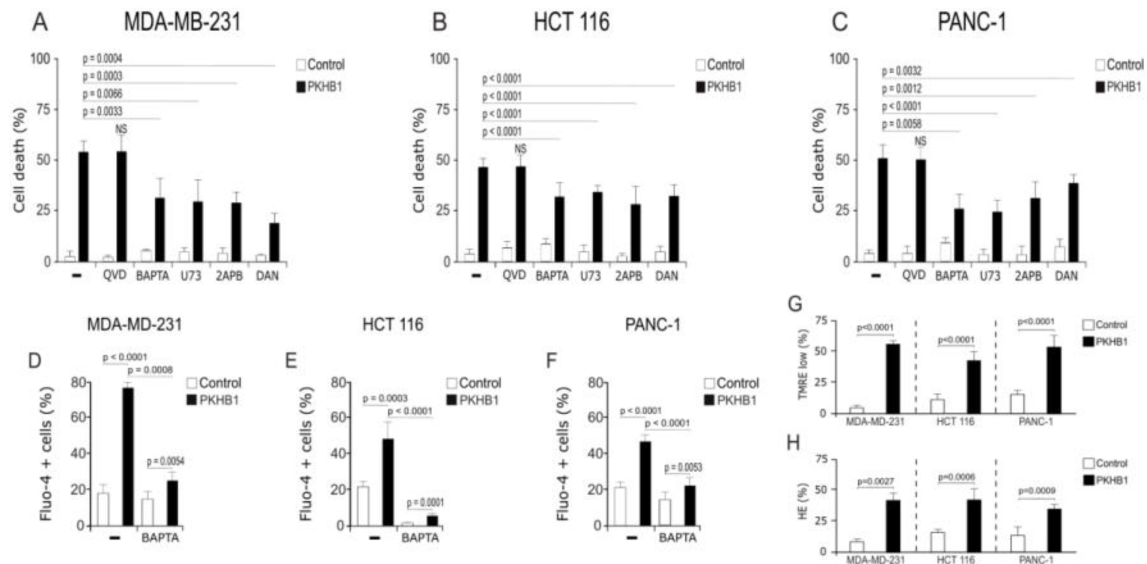

Figure 6. The cell death mechanism induced by PKHB1 is shared in breast, colon, and pancreatic cancer lines. PKHB1-induced cell death in MDA-MB-231 (6A), HCT 116 (6B) and PANC-1 (6C) cells was assessed by flow cytometry in the presence or absence (-) of the Ca^{2+} chelator BAPTA or the inhibitors, Q-VD-OPH (QVD), U-73122 (U73), 2-APB and Dantrolene (DANT). The increase in cytoplasmic Ca^{2+} was assessed by flow cytometry with the Fluo-4 AM probe in the presence or absence (-) of BAPTA in MDA-MB-231 (6D), HCT 116 (6E) and PANC-

1 (6F) cells. Loss of mitochondrial membrane potential (6G) and the presence of cytoplasmic ROS (6H) were assessed by flow cytometry with TMRE and HE labels, respectively. Graphs represent the average of at least three independent experiments performed in triplicate (± SD).

3. PKHB1 treatment induces extracellular Ca^{2+} influx, mitochondrial damage and increased cytoplasmic ROS.

Since it was observed that the cytotoxic effect induced by PKHB1 depends on the opening of ER Ca^{2+} channels (IP_3R and RyR) and extracellular Ca^{2+} entry, it was decided to evaluate cytoplasmic Ca^{2+} in the presence or absence of the extracellular Ca^{2+} chelator BAPTA, to determine whether the Ca^{2+} involved in cell death comes mostly from outside the cell or from Ca^{2+} stored in the organelles. Figure 6 shows MDA-MB-231 (6D), HCT 116 (6E) and PANC-1 (6F) cells treated with PKHB1, where it is observed that there is an increase in cytoplasmic Ca^{2+}, which is inhibited when using the chelator BAPTA, indicating that most of the Ca^{2+} involved in cell death comes from the extracellular medium.

The next step was to determine mitochondrial damage using the fluorescent dye TMRE, since it is attracted and retained by mitochondria with an intact membrane potential ($\Delta\Psi m$), so cells with loss of $\Delta\Psi m$ will show a lower fluorescence towards the labeling. The results of this assay are found in Figure 6G, where a higher percentage of cells with low fluorescence toward TMRE labeling is observed in the treated group compared to controls, indicating that PKHB1 induces mitochondrial damage.

Once mitochondrial damage was observed, we proceeded to evaluate the increase in cytoplasmic ROS using the compound hydroethidine (HE), which when reduced by ROS produces a fluorescent compound. The graphs of this assay are shown in Figure 6H, where it is observed that the cells treated with PKHB1 present a significant increase in cytoplasmic ROS.

4. PKHB1-treatment inhibits the migration of metastatic breast, colon, and pancreatic cancer cell lines even at sublethal concentrations.

Treatments with a cytotoxic and anti-metastatic capacity against tumor cells are needed to inhibit cancer recurrence (Ganesh & Massagué, 2021). In order to evaluate whether PKHB1 shows any effect on the metastatic capacity of breast, colon, or pancreatic cancer

cells, we first observed the effect of the peptide on the migration capacity of the cells by means of a scratch assay, where we used cytotoxic concentrations that were sublethal (CC_{SL}), induced 20% of cytotoxicity (CC_{20}) or induced 50% of cytotoxicity (CC_{50}).

Figure 7 shows a significant decrease in the migration capacity of MDA-MB-231 (7A), HCT 116 (7B) and PANC-1 (7C) cells at 24, 48 and 72 H when treated with PKHB1, highlighting that no significant difference is seen in the effect of CC_{SL}, CC_{20}, and CC_{50} in the three cell lines, where an average inhibitory effect of PKHB1 of approximately 38%, 32% and 42% is observed at the end point of the assay in MDA-MB-231 (7A), HCT 116 (7B) and PANC-1 (7C), respectively. Likewise, representative photos of the beginning and end of the assay in each group (Control, CC_{SL}, CC_{20} and CC_{50}) are shown in the MDA-MB-231 (7D), HCT 116 (7E) and PANC-1 (7F) cell lines, where it is observed how the cells treated with the different concentrations of the peptide continue to present an aperture while in the controls the scratch has already closed completely.

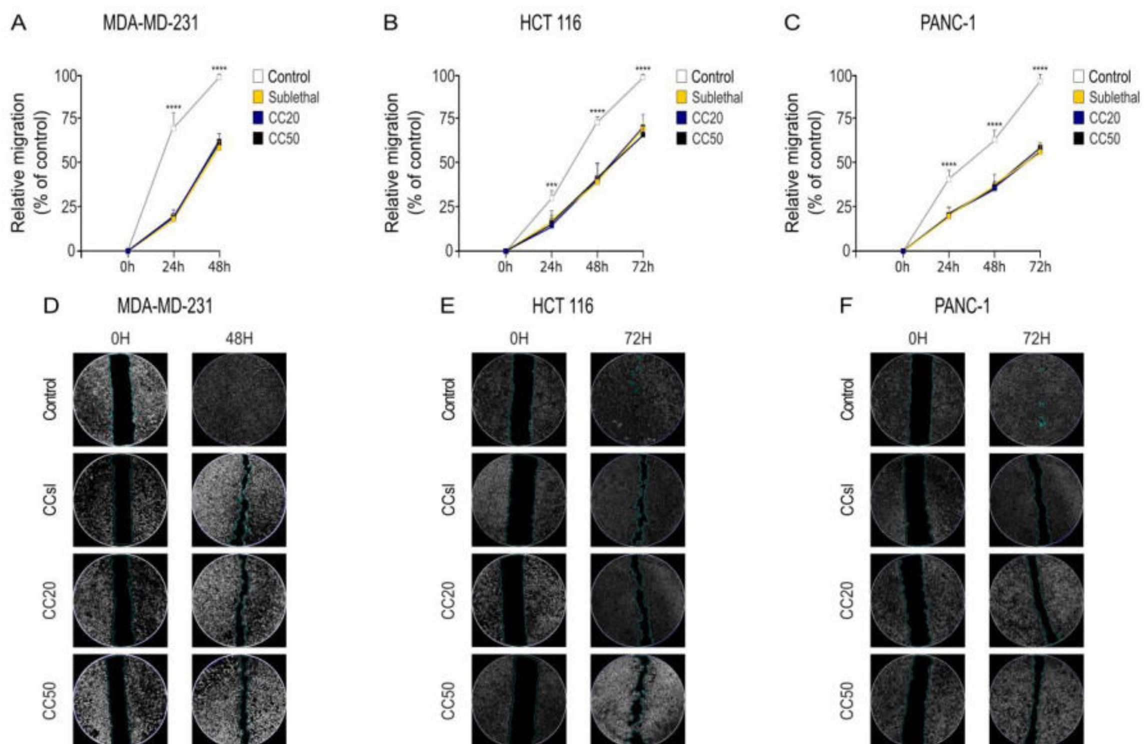

Figure 7. PKHB1 decreases the migration capacity of metastatic breast, colon, and pancreatic cancer lines. Cell migration capacity was evaluated by a scratch assay in control cells or cells previously treated with PKHB1 at CC_{SL}, CC_{20} and CC_{50} concentrations of each line, having

as end point of the assay a migration of approximately 100% in control cells. The upper part of the figure shows representative graphs of the percentage of migration of MDA-MB-231 (7A), HCT 116 (7B) and PANC-1 (7C) cells as time progresses. Subsequently, representative pictures of each group at time 0 and at the end point of the assay of MDA-MB-231 (7D), HCT 116 (7E) and PANC-1 (7F) cells are shown. The graphs represent the average of at least three independent experiments performed in triplicate (± SD).

5. PKHB1 reduces the invasiveness of metastatic breast, colon, and pancreatic cancer cell lines.

After having determined that PKHB1 is able to reduce the migration capacity of the MDA-MB-231, HCT 116 and PANC-1 lines, we proceeded to evaluate whether it was able to inhibit the invasiveness of the three cell lines used. For this purpose, invasion assays were performed with transwell chambers, where CC_{SL}, CC_{20} and CC_{50} were used.

Figure 8 shows that PKHB1 inhibits the invasion capacity of MDA-MB-231 cells by 40% (8A), HCT 116 by 30% (8B) and PANC-1 by approximately 43% (8C). It is noteworthy that the three concentrations used, either cytotoxic or not, show no statistical difference in the inhibition percentages in the three cell lines assessed.

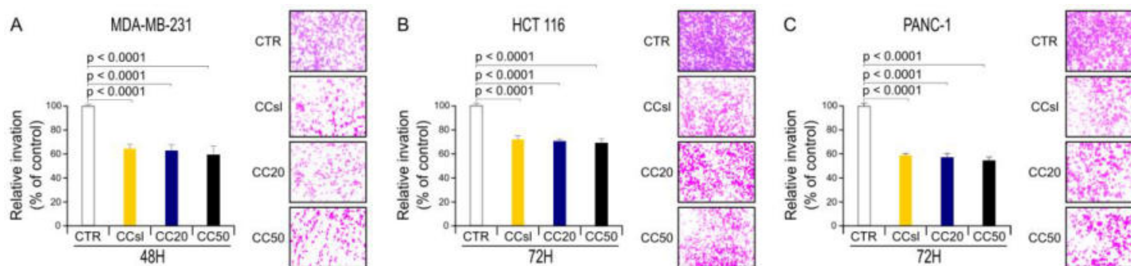

Figure 8. PKHB1 treatment inhibits the invasiveness of metastatic breast, colon, and pancreatic cancer lines. The inhibition of invasiveness was assessed by a transwell chamber assay. Graphs (left) and representative photos (right) of MDA-MB-231 (8A), HCT 116 (8B) and PANC-1 (8C) cells treated with PKHB1 at the CC_{SL}, CC_{20} and CC_{50} concentrations of each line are shown in the figure. Untreated cells were used as controls. Graphs represent the average of at least three independent experiments performed in triplicate (± SD).

6. PKHB1 decreases the replicative capacity of metastatic breast, colon, and pancreatic cancer cell lines.

After determining the effect of PKHB1 on the migration and invasion capacity of metastatic tumor lines, the last step was to evaluate the effect of the peptide on the replication capacity of cells that were not killed by the treatment. For this purpose, once the cells were treated with CC_{SL}, CC_{20} and CC_{50} concentrations, they were recovered and the number of viable cells was counted, then 100 viable cells were plated in a 6-well plate and left to replicate for 10 days. After 10 days, the cells were stained and counted.

Figure 9 shows the results of this assay, where it is observed that the effect of PKHB1 on the replicative capacity of the cells is dose-dependent, with the MDA-MB-231 line showing the lowest sensitivity with an inhibition of 79% with CC_{SL}, followed by 47% with CC_{20} and 34% with CC_{50}. On the other hand, the HCT 116 and PANC-1 lines showed similar inhibition percentages with 56 and 60% with CC_{SL}, 42 and 43% with CC_{20} and 27 and 28% with CC_{50}, respectively.

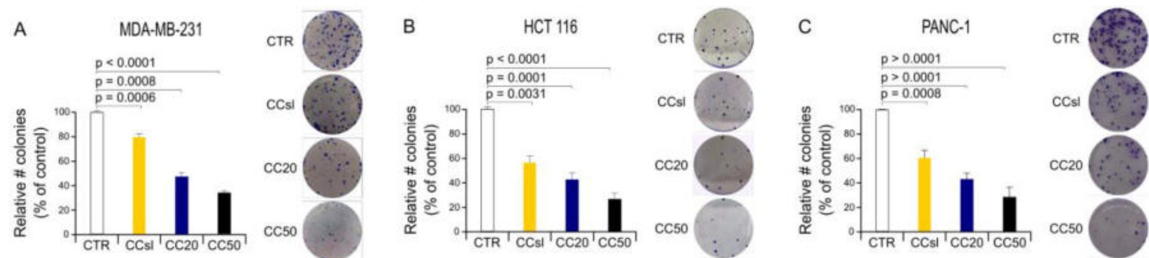

Figure 9. PKHB1 treatment reduces the replicative capacity of metastatic breast, colon, and pancreatic cancer lines. Inhibition of replication capacity was assessed by a clonogenicity assay. The graphs (left) and representative photos (right) of MDA-MB-231 (9A), HCT 116 (9B) and PANC-1 (9C) lines treated with PKHB1 at the CC_{SL}, CC_{20} and CC_{50} concentrations are shown in the figure. Untreated cells were used as controls. Graphs represent the average of at least three independent experiments performed in triplicate (± SD).

DISCUSSION

In this thesis it was demonstrated that PKHB1 induces cell death in metastatic breast (MDA-MB-231), colon (HCT 116) and pancreatic (PANC-1) cancer lines. Finding a CC_{50} of 200µM in the MDA-MB-231 cell line, corresponding with that observed by Calvillo-Rodriguez et al (2022), and 250µM in the HCT 116 and PANC-1 lines. In this regard, Uscanga-Palomeque et al (2019) report that murine T-cell acute lymphocytic leukemia L5178Y-R cells also show a CC_{50} of 200µM, while Calvillo-Rodriguez et al (2022) report a CC_{50} of 300µM in the murine breast cancer line 4T1, so the sensitivity of the HCT 116 and PANC-1 lines remains within the range of efficacy reported for PKHB1.

These differences in the sensitivity of the cell lines may be due to the molecular characteristics of each cell type, mainly those related to the PKHB1-induced cell death pathway, since, for example, it has been observed that the MDA-MB-231 line presents an overexpression of the Orai1 protein (Jardin et al, 2022), while T-cell acute lymphocytic leukemia cells show an overexpression of PLC (Pan et al, 2018). On the other hand, no overexpression of major proteins related to the PKHB1-induced cell death pathway has been reported in 4T1, HCT 116 and PANC-1 lines.

This is verified with the measurement of intracellular Ca^{2+}, where it was determined that most of the Ca^{2+} comes from the extracellular medium, possibly due to the activity of channels involved in the calcium entry by calcium exit pathway such as Orai and TRP channels (Monteith et al, 2017), but even chelating this extracellular Ca^{2+} there is still a significant increase in intracellular Ca^{2+} compared to the control, so there is a release of Ca^{2+} stored in the organelles. Likewise, it was observed that there is mitochondrial damage and an increase in cytoplasmic ROS, which can also be related to an outflow of Ca^{2+} from the ER, since this can lead to an increase in mitochondrial Ca^{2+} inducing damage and stress in this organelle leading to the overproduction of ROS (Baumgartner et al. 2009).

Intracellular Ca^{2+} assessment shows that most of the Ca^{2+} comes from the extracellular medium, possibly due to the activity of channels involved in the store-operated Ca^{2+} entry pathway such as Orai and TRP channels (Monteith et al, 2017). However, even when this extracellular Ca^{2+} is chelated there is still a significant increase in intracellular Ca^{2+}

compared to the control, which indicates a release of Ca^{2+} stored in the organelles. Likewise, it was observed that there is mitochondrial damage and an increase in cytoplasmic ROS, which can also be related to an outflow of Ca^{2+} from the ER, as this can lead to an increase in mitochondrial Ca^{2+} inducing damage and stress in this organelle leading to overproduction of ROS (Baumgartner et al. 2009).

It has been reported that cancer cells present deregulations in Ca^{2+} signaling pathways (Zheng *et al*, 2022). This can be observed since the development of cancer cells, where each of the hallmarks of these cells are directly related to these calcium dysregulations (Stewart et al. 2015). Furthermore, specific oncogenic and pro-oncogenic pathways that confer resistance to apoptosis, as well as, signaling leading to the epithelial-mesenchymal transition (EMT) phenotype, cancer stem cell production, and tumor cell migration and invasion are modulated by Ca^{2+} signaling pathways (Monteith et al, 2017). Among the different mutations observed on this signaling pathway, overexpression of proteins such as: PLC-γ, plasma membrane cation channels (members of the TRP, Cav, ORAI and PMCA families) and ER calcium channels (members of the IP_3R, SERCA families) stand out (Monteith et al. 2012).

A sign of the dependence of these cells on Ca^{2+} deregulation is that it has been reported that the loss of functionality or production of proteins such as PLC, Orai1 and TRP can decrease tumor growth and significantly reduce the metastatic capacity of tumor cells (Diez-Bello et al, 2019, Nunia et al, 2023). Thus, the fact that our peptide uses these dysregulations to perform a cytotoxic effect, coupled with the fact that the loss of these proteins reduces the tumorigenicity of cancer cells, makes our treatment an object of study of great importance. Even more when we observed that the mechanism of cell death induced by the peptide is conserved among different types of cancer (Martinez-Torres et al, 2015, Calvillo-Rodriguez et al, 2022, Uscanga-Palomeque et al, 2019), including the most aggressive and metastatic subtypes as seen in this thesis.

Similarly, in this thesis it was observed for the first time that PKHB1 has an effect on the main metastatic processes, reducing the migration and invasion capacity of MDA-MB-231, HCT 116 and PANC-1 cells by approximately 40%, 32% and 43%, respectively, without significant differences between CC_{SL}, CC_{20} and CC_{50}. It should be emphasized

that the fact that this effect is observed from the CC_{SL} is an indication that it is really due to an inhibition of the migration and invasion processes and not due to the cytotoxic effect of the peptide *per se*. Likewise, PKHB1 was shown to inhibit the replicative capacity of MDA-MB-231, HCT 116 and PANC-1 cells in a dose-dependent manner, having its lowest effect in the MDA-MB-231 line with a 20% inhibition at CC_{SL}, reaching up to a 65% inhibition at CC_{50}. On the other hand, the peptide induced an inhibition of approximately 60% in the HCT 116 and PANC-1 lines at CC_{SL} and inhibitions of 74 and 72% at CC_{50}, respectively.

These anti-metastatic effects may be due to different reasons:

Firstly, it has been shown that the peptide 4N1K (precursor of PKHB1) inhibits VCAM expression in endothelial cells through its interaction with CD47 (Soriano-Romani et al 2022), which is of great importance, since it has been reported that a knockout (KO) of VCAM inhibits the migration and invasion capacity of breast, colon, and lung cancer cells (Zhang et al, 2020).

On the other hand, it has been seen that calcium entry through the store-operated Ca^{2+} entry pathway can induce negative feedback leading to loss in TRPC6 expression, culminating in inhibition of tumor cell migration and invasion (Jardin et al,2018). This effect was seen on MDA-MB-231 and MCF-7 cells with Oleocanthal from a CC_{20} inducing an inhibition of approximately 67%, however with this treatment no inhibition was seen when using a sublethal concentration (Diez-Bello et al, 2019) unlike what we observed with PKHB1.

Similarly, it has been described that TSP-1 manages to inhibit tumor growth through its interaction with Fyn src kinase, which also involves the interaction with CD47 (Lawler, 2002). In this sense, it has been shown that TSP-1/CD47 interaction can generate senescence in endothelial cells (Bitar, 2019), as well as inhibit the proliferation of murine ovarian cancer epithelial cells (Jeanne et al, 2015) and prostate cancer cells in vivo and in vitro (Fang et al, 2016).

Finally, Martinez-Torres et al (2015) reported that the mechanism of cell death induced by PKHB1 on CLL cells induces actin depolymerization, which is interesting since it has

been reported that the formation of actin filaments is necessary to carry out processes such as protein transcription, so it has been seen that inducing actin depolymerization stops the production of proteins reaching to see an arrest in the cell cycle (Izdebsca et al, 2018). This effect has been observed in treatments such as Timosaponin AIII (TSAIII), which induces an inhibition of cell replication, as well as inhibition of cell migration and invasion, as these processes also require the formation of actin filaments. However, this treatment does not show any inhibitory effect at sublethal doses (Hsieh et al, 2021) unlike that observed with PKHB1.

CONCLUSION

With the results of this thesis we can conclude that the mechanism of cell death induced by PKHB1 on metastatic breast, colon and pancreatic cancer cells is characterized by being different from apoptosis and by presenting an increase in cytoplasmic Ca^{2+}, which starts with a depletion of the Ca^{2+} stored in the ER through the PLC/IP_3 pathway, promoting an influx of extracellular Ca^{2+} through store-operated Ca^{2+} channels, followed by mitochondrial damage and overproduction of ROS that culminates in cell death. Therefore, the mechanism of cell death induced by the peptide is conserved among different types of cancer, even in the most aggressive and metastatic subtypes. Likewise, it was proven that PKHB1 reduces the metastatic capacity of breast, colon and pancreatic cancer cells, inhibiting cell migration and invasion from sublethal concentrations, without a significant difference between the doses used, as well as inhibiting cell replication from sublethal concentrations, but in a dose-dependent manner.

PERSPECTIVES

- To determine whether PKHB1 induces actin filament depolymerization in metastatic cells.

- To evaluate the presence of TRPC6 in viable cells after treatment with PKHB1, to see if there is a decrease in its expression

- Determine whether PKHB1 treatment inhibits VCAM1 expression.

- To analyze whether PKHB1 has a cytotoxic effect on cancer stem cells by evaluating the expression of molecular markers such as ALDH1, CD24 and CD44 in tumor cells after treatment with the peptide.

- To evaluate the anti-metastatic effect of PKHB1 in vivo

- To analyze the effect of PKHB1 on cancer cells that have undergone a metastatic process, by isolating tumor cells in the main metastatic sites of tumor-bearing mice and subsequently evaluating their sensitivity to PKHB1.

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