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Facultat de Farmàcia
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Final degree project

NEW THERAPIES TARGETING THE PD1/PDL-1 PATHWAY FOR LUNG CANCER

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ABSTRACT:

Lung cancer is giving promising results in immunotherapy treatments, even though it is one of the cancers with worse prognosis. Silencing of the PD1 / PDL-1 pathway is one of the most widely used strategies for this type of cancer. This pathway is usually found to be overexpressed in lung cancer; it should be noted that it helps tumor cells to evade the anti-tumor response of the immune system. PPRHs are DNA molecules used for gene silencing. They were recently discovered and have been previously tested in other types of cancer to modulate the PD1 / PDL-1 pathway. During the present research project, the main objective was to validate the PPRHs strategy to inhibit the PD1/PDL-1 pathway as a possible treatment of pulmonary adenocarcinoma. THP-1 cells were differentiated to macrophages with PMA. Different PPRHs against PD1 and PDL-1 were designed and tested in THP-1 and A549 cell lines, first separately, testing for cross toxicity and then in co-culture. Cell viability was assessed by MTT assays. Differentiation of THP-1 to macrophages showed no significant differences at three different concentrations of PMA. Cross-toxicity assays showed a decrease in cell viability when transfecting A549 cells with PPRHs against PD1, whereas this effect was not observed in THP-1 transfected with PPRHS against PDL-1. Co-culture experiment showed that the combination of PPRHs against PD1 and PDL-1 produced an enhancement of the antitumor effect, compared to their individual effects separately. Our results support the usage of PPRHs for the silencing PD1/PDL-1 pathway in lung cancer.

Keywords: Immunotherapy, PD1, PDL-1 PPRHs

RESUM:

El càncer de pulmó es un dels càncers amb pitjor pronòstic, però a l'hora es un dels que millor respon a la immunoteràpia. Una de les estratègies d'immunoteràpia contra aquest càncer és el silenciament de la via PD1/PDL-1, la qual es troba sobreexpressada en els càncers de pulmó. Recentment, s'ha descobert una nova tecnologia per silenciar gens, els PPRHs, els quals han estat prèviament assajats per inhibir la via de PD1/PDl-1 en altres tipus de càncer. En aquest projecte, l'objectiu principal ha sigut validar la tecnologia dels PPRHs com a possible tractament de l'adenocarcinoma pulmonar, mitjançant el silenciament de la via PD1/PDL-1. Les cèl.lules THP-1 van ser diferenciades a macròfags utilitzant tres concentracions diferents de PMA. Es van dissenyar PPRHs contra PD1 i PDL-1 i es van transfectar creuadament a les cèl.lules A549 i THP-1. També es va realitzar un co-cultiu amb cèl.lules THP-1 diferenciades i A549 en presència dels PPRHs contra PD1 i/o PDL-1. La viabilitat cel·lular es va determinar mitjançant assaigs de MTT. La diferenciació de THP-1 a macròfags no va mostrar diferencies significatives a les diferents concentracions de PMA. En els assaigs de toxicitat creuada es va observar que la viabilitat de les cèl.lules A549 disminuïa amb anti-PD1. A l'experiment de co-cultiu es va observar com la combinació de PPRHs contra PD1 i PDL-1 produïa una potenciació de l'efecte antitumoral. Finalment vam poder concloure que els PPRHs contra PD1 i PDL-1 podrien ser una estratègia valida contra el càncer de pulmó.

Paraules clau: Immunoteràpia, PPRH, PD1, PDL1

ABREVIATIONS

A549: Lung Adenocarcinoma cell line

aODNs: Antisense Oligonucleotides

APC: Antigen Presenting Cell

CNT: Control

CTLA4: T-Lymphocyte-Associated protein 4

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DOTAP: N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methylsufate

E1: Exon 1

EDTA: Ethylenediaminetetraacetic acid

FDA: Food and Drug Administration

I1/2/4: Intron 1/2/4

IASLC: International Association for the Study of Lung Cancer

IFN-α: Interferon α

IL-2: Interleukin 2

MHC I/II: Major Histocompatibility Complex I/II

miRNA: MicroRNA

MTT: 3- (4,5-Dimethylthiazol-2-yl) -2,5-Diphenyl-tetrazolium Bromide

NCHS: National Center for Health Statistics

NCI: National Cancer Institute

NSCLC: Non-Small Cell Lung Cancer

PBS: Phosphate Buffered Saline

PD1: Programmed cell Death 1

PDL-1/2: Programmed cell Death Ligand 1/2

PMA: Phorbol 12-Myristate 13-Acetate

PPRH: Polypurine Reverse Hoogsteen

PR: Promotor

RNA: Ribonucleic Acid

SCLC: Small Cell Lung Carcinoma

siRNA: Small interference RNA

TCR: T-Cell Receptor

TFOs: Triplex Forming Oligonucleotides

THP-1: Human Monocyte Leukemia cell line

WHO: World Health Organization

DISCUSSION JUSTIFYING WORK INTEGRATION OF THE AREAS INCLUDED IN THIS PROJECT:

In the present project, various areas of the studies of Pharmacy have been integrated. First of all, it was necessary to have knowledge of the field of Physiology and Physiopathology, because in this project we studied a disease, lung cancer. Secondly, Pharmacology knowledge has been integrated, since in this case we were looking for therapeutic targets to treat pulmonary adenocarcinoma. The treatment studied has been immunotherapy, so it has also been necessary to appeal to Immunology area to develop this research. Lastly, the fields of Molecular Biology and Biochemistry were involved, especially in the practical phase of the project, since this knowledge has been necessary for the design and development of all the experiments.

1. INTRODUCTION:

1.1 Cancer disease:

The term cancer involves a collection of more than 200 related diseases that can start almost anywhere in the human body. These diseases are induced by a group of cells that have experienced one or more mutations. All these cells have two main aspects in common: an uncontrollable proliferation and the ability to spread into surrounding tissues (1).

In the last decades, cancer has become a worldwide major health issue. According to the National Center for Health Statistics (NCHS) 1762 450 new cancer cases and 606 880 cancer deaths were expected in 2019 (2).

Of all types of cancer, the one that leads to the highest number of deaths is lung cancer, which represents more than 20% of total cancer deaths. This is the reason to focus our study on this type of cancer (2).

1.2 Lung cancer:

Bronchogenic carcinoma, also known as lung cancer, comprehends all types of malignant tumors of epithelial lines, which originate from the lining or glandular epithelium of the bronchial tree (3).

1.2.1 Classification:

As claimed by the World Health Organization (WHO) and the International Association for the Study of Lung Cancer (IASLC), two major types of lung cancer can be distinguished: Small Cell Lung Carcinoma (SCLC) and Non-Small Cell Lung Carcinoma (NSCLC). As it can be seen, the name given to each of the types refers to the size of the cells that conform the tumor (4).

SCLC represents 20% of all lung cancers. This type is the most aggressive one and also the one with worst prognostic, attributable to its greater tendency to

spread to other organs. SCLC tumors are composed by small cells with scarce cytoplasm presenting neurosecretion granules on it, they are usually oval, round or fusiform. Two main characteristics of this kind of tumors are its high mitotic rate and the abundant necrosis (4).

The remaining 80% of lung tumors are classified as NSCLC. According to the NCHS, NSCLC usually have a better prognostic and a lower death rate, due to a greater response to treatments and slower cell division. Cells that conform this type of lung cancer are bigger than in SCLC, with a greater amount of cytoplasm, but without neurosecretion granules on it, and also visible nucleoli (2,4).

The Non-Small Cell Lung Cancer type can be divided in 3 subcategories: Squamous Cell Carcinoma, Adenocarcinoma and Large Cell Carcinoma, being the Adenocarcinoma the most common one, approximately 40% of cases, followed by Squamous Cell Carcinoma and finally Large Cell Carcinoma (5).

1.2.2 Symptomatology and diagnosis:

The main problem of lung cancer is that it does not present a clear symptomatology until it reaches advanced stages of the disease, which explains why, nowadays, there is no early diagnosis (6).

Depending on the location of the tumor and the stage of the disease, different signs and symptoms can be observed. The most frequent ones are cough, usually with expectoration, hemoptysis, dyspnea and thoracic pain. In advanced stages of the disease more symptoms can be noticed, such as bone pain, tiredness, anorexia and others related to possible metastases (6).

Lung cancer diagnosis can be performed using different types of tests, some of them more invasive than others. The non-invasive ones are chest X-ray, sputum cytology, spirometry, computed tomography and positron emission tomography, this last being the most sensitive. Other available tests, such as bronchoscopy and fine needle puncture, are considered as invasive techniques (5,6).

1.2.3 Prognostic:

As stated by the Spanish Association Against Cancer, lung cancer survival after 5 years is less than 15%, becoming one of the cancers with worst prognostic, along with liver, esophagus and pancreas. If patients diagnosed with advanced stage do not receive treatment, their life expectancy does not exceed 6 months. However, it has been observed that diagnosed patients in asymptomatic stages showed an increase up to 70% in the survival rate after 5 years (7).

1.2.4 Treatments:

The treatment to choose for lung cancer depends on different factors such as the anatomopathological classification, SCLC or NSCLC, in addition to the stage of the disease, either initial and localized or advanced with metastasis.

In the case of SCLC, if the patients are diagnosed with a limited disease, the treatment will probably be a combination of chemotherapy and radiotherapy. However, when the disease is widespread, palliative chemotherapy is given, since its rarely curable (8).

On the other hand, when it comes to NSCLC, if the disease is found in early stages (localized disease) the treatment of choice will be surgery. However, only 24% of cases are diagnosed in early stages, which implies that most of patients will have locally advanced or disseminated disease, and in these two scenarios, chemotherapy and/or radiotherapy will be the chosen treatments, whereas surgery will be the second option (5).

Although surgery, chemotherapy and radiotherapy are conventional treatments, there are many other treatments that can be used against lung cancer, such as intrabronchial laser, radiofrequency, molecular targeted therapy and immunotherapy (5,8).

In the last decade, immunotherapy has been gaining ground over other treatments, due to its surprising and durable results, in addition to its low toxicity rate and its few side effects (9).

1.3 Immunotherapy:

The use of a therapy that is capable of boosting the patient's immune system, to help them fight a disease such as cancer, with fewer off-target effects, represented a complete revolution in oncology and research on this field has not stopped growing (9,10).

The history of this therapy starts back in 1980, when the use of cytokines to treat some types of cancer locally and systematically was found out, although the great revolution took place in 2011 with the discovery of checkpoint inhibitors (11).

The idea of immunotherapy relies on the insight that the immune system, in a process named immune surveillance, can eliminate cancerous cells during its initial transformation. During oncogenesis, tumors create neo-antigens, which should turn these cells into a target for the immune system. But the reality is that neoplastic cells are able to escape from the immune system, due to the different resistance systems they create, such as induction of tolerance, local immune evasion and systemic disruption of T cell signaling (10).

1.3.1 Types of immunotherapy:

There are many types of cancer, and accordingly different types of immunotherapy, which include various approaches, from stimulating effector mechanisms to counteracting suppressor and inhibitory mechanisms (9,10). The different types of existing immunotherapies are summarized below:

Strategy	Type	Principal Mechanism
Cytokines	IL-2	Stimulating host's IS.
	IFN-α	Stimulating host's IS.
Cell based therapies	Vaccines	Stimulating host's IS.
	Adoptive cellular therapy	High avidity in effector T cells and omits the task of breaking tolerance to tumor antigens.
Immune checkpoint blockade	CTLA-4	Unleash pre-existing anticancer T cell responses and possibly triggers new ones.
	Anti PD1/ PDL-1 antibodies	Enhances T cell response and mediate antitumor activity.
	Combination immunotherapy	Improves anti-tumor responses and immunity.

Table 1: Summary of existing types of immunotherapy and examples (10).

1.3.2 Immunotherapy and Lung Cancer:

As previously described, lung cancer is one of the most aggressive type of tumors, with the worst prognosis and responsible for the greatest number of deaths in the world, but surprisingly it is also one of the types of cancer that best responds to immunotherapy treatment (12).

It is worth mentioning that lungs are the way of entry for many external agents, and thus they are rich in various types of immune cells. Furthermore, pulmonary oncogenesis is often caused by inflammatory alterations due to immune defects such as T cell alterations, B-Lymphocyte defects, natural killer cell dysfunction and defective dendritic cell, monocyte and neutrophil functions (13).

The use of immunotherapy is being especially limited to patients diagnosed with advanced stages of NSCLC, although more recently, testing has also begun in SCLC cases (8). Even so, in this work we will focus on NSCLC.

For the reasons mentioned above, considerable time is currently being spent on researching new treatments for lung cancer based on the exploitation of the patient's immune system. There are currently two different groups of immunotherapies against this type of cancer: passive and active immunotherapy (12).

Passive immunotherapy can be divided into two types, dendritic cell based and adoptive transfer of ex-vivo-activated T-cells. The antitumor activity of both is based on the incorporation of components of the immune system, either from donors or performed in the laboratory, to the patient (12).

On the other hand, active immunotherapy can be divided into two types, antigen-dependent immunotherapy, which it is based on the use of vaccines, and antigen-independent immunotherapy, which works by blocking immune checkpoints (12).

It should be noted that presently, the type of immunotherapy with best results against lung cancer is the active antigen independent (Checkpoint Blockade) (14).

1.3.3 Checkpoint Blockade:

The most important effector cells against tumors are CD8+ cytotoxic and CD4+ helper T cells, since both are responsible for mediating humoral and cell-mediated responses. The activation of T lymphocytes begins when Antigen Presenting Cells (APC), through their Major Histocompatibility Complexes (MHC-I / MHC-II) present antigens to T lymphocytes, which recognize them by their receptor (TCR). Complete activation, differentiation and proliferation occurs thanks to other costimulatory factors such as CD28 (15,16).

The immune system is self-regulated through co-stimulatory and co-inhibitory molecules, with the aim of maintaining homeostasis. Both strategies are equally important, since the first one allows to obtain an efficient response against possible pathogens, whereas the second one controls that this response is not excessive (16).

T lymphocytes, therefore, also have their own regulatory molecules, the most prominent being the co-inhibitory molecules of the immune checkpoint, in charge of regulating both the duration and the amplitude of the response of T cells. These co-inhibitory molecules comprehend T-lymphocyte-associated protein 4 (CTLA4), programmed cell death 1 (PD1), and programmed death ligands (PDL-1 / PDL-2) (11,17).

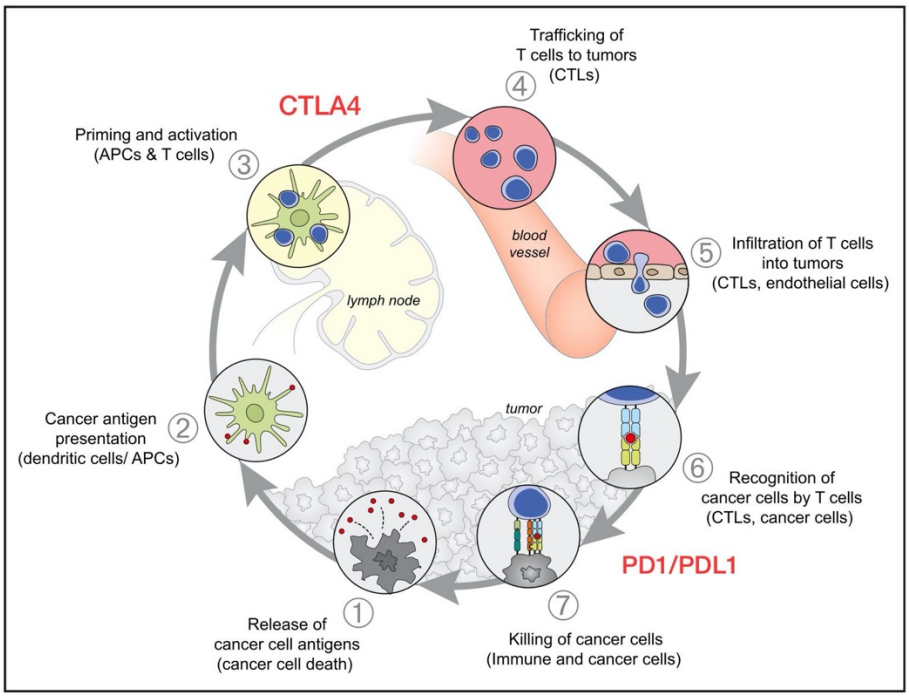

Figure 1: Cancer Immunity (Modified Image) (11).

As mentioned above, cancer cells are able to evade the immune system, and, in the case of NSCLC, it has been observed that resistance mechanisms are due to inhibitory molecules located in the tumor microenvironment. Among all inhibitory molecules, two types should be highlighted: the immune inhibitory ligand-receptor interaction, which are related to the CTLA4 and PD1, and the

membrane inhibitory ligands (also known as checkpoint ligands) which are PDL-1 / PDL-2 (14,16).

- CTLA4:

The inhibitory molecule CTLA4 is found on the surface of activated T lymphocytes and competes with CD28 (costimulatory molecule) to bind to its ligands. CTLA4 is in charge of regulating the amplitude of the T cell response (11,16).

In patients with NSCLC, the expression of CTLA4 is increased, and thus the response of T lymphocytes will be directly reduced, since it can bind more easily to ligands, causing a decrease in the activation of T cells, promoting a downregulation of antitumor immune response (14,16).

For this reason, antibodies as possible inhibitors of this molecule have been studied, in order to boost the anti-tumor response of T cells. It should be noted that, although the response of these antibodies has been good in combined therapy, treatments against PD1 and PDL-1 have been shown to be more effective in NSCLC (11,14,16).

- PD1/PDL-1:

The PD1 receptor can be found on the surface of various cells of the immune system, including T or B lymphocytes and Natural Killer. Right after antigen recognition, T cells express PD1 on their surfaces. This molecule has two ligands PDL-1 and PDL-2, which can be found in different cell lines. PDL-1 expression can be induced both by the presence of inflammatory cytokines and by oncogenes, which explains PDL-1 overexpression in various tumors, especially NSCLC (15,16).

The binding of PD1-PDL-1 causes a downregulation of the immune system, since it decreases the production of inflammatory

cytokines and cytotoxic activity, and will end up causing the death of T lymphocytes, and thus promoting an escape route for tumor cells (16,18).

In various studies, it has been observed that the blockade of this pathway leads to an increase in effector T cells and in the cytotoxic activity of tumor-specific T cells, enhancing the activity of tumor infiltrating T cells and T regulatory cells, boosting the immune system (15).

For this reason, in recent years, studies have focused on the search for monoclonal antibodies capable of inhibiting the binding of PD1/PDL-1, either by blocking PD1 receptors or its PDL-1 ligand. In 2017, two anti PD1 antibodies (Nivolumab and Pembrolizumab) and 3 anti PDL-1 (Atezolizumab, Avelumab and Durvalumab) were approved by the Food and Drugs Administration (FDA) (14,18).

The pharmacological profile of these monoclonal antibodies is highly favored, due to their low side effects, which are usually mild and revert when treatment is suspended, in addition to their advantages due to their good antitumor activity and their long-lasting responses (15).

1.4 PPRHs technology:

There are certain diseases whose treatment are based on gene silencing, decreasing expression of certain genes. The most used molecules to produce gene silencing are the following: small-interference RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (aODNs) and Triplex Forming Oligonucleotides (TFOs) (19).

In recent years, a new technology called Polypurine Reverse Hoogsteen Hairpins (PPRHs) has emerged, which does not have some of the drawbacks of the

above-mentioned molecules, such as low stability and the response of the immune system (19).

PPRHs are DNA hairpins consisting of two antiparallel polypurine domains, without nucleotide modifications. These strands are linked by a pentathyamidine loop, which allows the formation of intramolecular reverse Hoogsteen bonds. Hairpins can be linked by Watson and Crick bounds to the DNA, provided that it has polypirimidine targets, which are usually found in promoters and introns. Targets for PPRHs can be found in both of the DNA strands, meaning that PPRHs can be directed against either the coding or the template strands. When PPRHs bind to the DNA, they cause a strand displacement, inhibiting transcription (19–21).

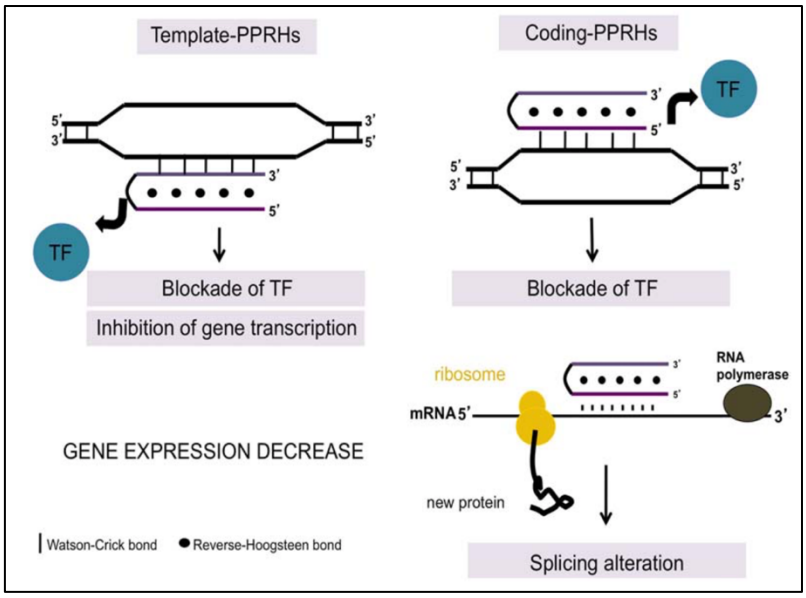

Figure 2: Template and coding PPRHs (19).

The effectiveness of PPRHs in cancer immunotherapy has been tested in different cell lines, targeting different genes related to the immune system. The use of this technology against the PD1/PDL-1 tandem has been previously tested in prostate, cervix, breast and melanoma cancer cells, in which a decrease in mRNA and protein levels of both genes have been observed. In addition, in co-culture experiments with PDL-1-transfected tumor cells, together with PD1-transfected macrophages, a reduction of at least 65% in the viability of the tumor cells could be observed (20,21).

In the present work, our aim is to evaluate the silencing PD1 and PDL-1 by means of the PPRH technology in lung adenocarcinoma cells.

2. OBJECTIVES:

During the present research project, the main objective has been to validate Polypurine Reverse Hoogsteen strategy as a possible treatment of pulmonary adenocarcinoma, by silencing the PD1/PDL-1 pathway.

3. MATERIALS AND METHODS:

3.1 Cell culture

Adenocarcinoma A549 cells, representative of the most common type of tumor within non-Small Cell Lung Cancer, and THP-1 cells (Human Monocyte Leukemia) commonly used as a model of macrophages were the cell lines used in this work.

Culture of both cell lines was performed using Ham's F-12 medium supplemented with 10% fetal bovine serum (both from Gibco, Barcelona, Spain). Cell culture conditions were as follows: temperature of 37ºC in a humidified 5% CO_2 controlled atmosphere. A549 cells grow attached, so it was necessary to perform trypsinizations, for which it was used 0.05% trypsin in PBS 1X (154 mM NaCl, 3.88 mM H2NaPO4 and 6.1 mM HNaPO4 pH 7.4) (Sigma-Aldrich, Madrid, Spain). THP-1 cells grow in suspension, so no trypsinization was needed.

Both A549 and THP-1 were maintained frozen in liquid N_2 tanks at approximately -150° C, so it was necessary to thaw them first. Once thawed, they were centrifuged in order to eliminate the freezing medium (with a high content of dimethyl sulfoxide (DMSO), toxic for the cells) and were subsequently resuspended with Ham's F-12 medium supplemented with 10% fetal bovine serum.

A549 and THP-1 cells were plated in cell culture dishes and incubated for 24 hours under optimal growth conditions (37 ºC, 5% CO_2 controlled humified atmosphere) to ensure their viability, before any experimental procedure.

3.2 THP-1 Differentiation

Differentiation of THP-1 cells to macrophages was performed in the presence of 3 different concentrations (1, 2 and 3 ng/ml) of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Madrid, Spain) dissolved in DMSO. After 72 hours of incubation under optimal conditions (37 ºC, 5% CO_2 controlled humified atmosphere), adhesion of the cells to the dishes was visually evaluated, as a control parameter for differentiation.

3.3 Design of PPRHs

The PPRHs used in the present co-culture experiment were designed using the Triplex Oligonucleotide Target Sequence Search Software (http://utw10685.utweb.utexas.edu/tfo/, Austin, Texas, USA), a program capable of finding polypurine sequences in the genes of interest.

The sequences of the PPRHs against PD1 and PDL-1 and their abbreviations are shown below:

PPRHS AGAINST PD1		
Name	**Sequence (5'-3')**	**Position**
HPPD1-PR	GAGCAGAGACACAGAGGAGGAAGGG T T T GAGCAGAGACACAGAGGAGGAAGGG T T	Promoter
HPPD1-E1	AGGCGGAGGTGAGCGGAAGGGAAA T T T AGGCGGAGGTGAGCGGAAGGGAAA T T	Exon 1
HPPD1-I4	GAGGAGAAAGGGAGAGGGAG T T T GAGGAGAAAGGGAGAGGGAG T T	Intron 4

Table 2: Sequences of the PPRHs against PD1.

PPRHS AGAINST PDL-1		
Name	**Sequence (5'-3')**	**Position**
HPPDL-1-I1	GGGATGGAGAGAGGAGAAGGGAAAGGGAA T T T GGGATGGAGAGAGGAGAAGGGAAAGGGAA T T	Intron 1
HPPDL-1-I2	AGTGGTGAAGGGAGGAGGGACA T T T AGTGGTGAAGGGAGGAGGGACA T T	Intron 2

Table 3: Sequences of the PPRHs against PDL-1.

The synthesis of the PPRHs was carried out by Sigma Aldrich (Haverhill, United Kingdom). PPRHs were resuspended in TE buffer (1mM Ethylenediaminetetraacetic acid (EDTA) and 10mM Tris, pH 8) and stored at -20ºC until their use.

3.4 Transfection of PPRHs

Transfection of PPRHs was carried out using DOTAP (N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methylsufate) (Biontex, München, Germany), a delivery system capable of transferring DNA to cultured cells. This system was previously used in other studies, in which it was observed that the optimal ratio between DOTAP and PPRH was 1:100 (22).

The mixture used for the transfection was 100 nM of PPRH with 10 µM of DOTAP in a 200 µl final volume. The final 200 µl solution was incubated at room temperature for 20 minutes. Subsequent to said incubation, the 200 µl of solution were added into the appropriate well, obtaining a final volume of 1 ml in each.

3.5 Cross-citotoxicity experiments

Cross-citotoxicity experiments were carried out by plating 6 well-dishes with THP-1 and A549 cells separately, and later transfected with the corresponding PPRHs, THP-1 cells were transfected with PPRHs against PDL-1 (I1 and I2) whereas A549 cells were transfected with PPRHs against PD1 (I4, E1 and PR). Cells were incubated for 4 days,

under optimal conditions (37 ºC, 5% CO_2 controlled humified atmosphere) and on the fourth day, cell viability was determined by MTT assays.

3.6 Co-culture experiments

First, THP-1 were plated in 6-well dishes and transfected with PPRHs against PD1 (I4, E1 and PR). After 24 hours of transfection, the process of differentiation of THP-1 into macrophages began, by adding 2 ng/ml of PMA to the culture medium.

After incubating the dishes for 72 hours, cells were already differentiated into macrophages. Then the culture medium with the remaining PMA was aspirated, and A549 cells were plated together with the macrophages and incubated for 6 hours, to ensure their correct adhesion to the plate. After this time period, tumor cells were transfected with PPRHs against PDL-1 (I1 and I2).

Co-cultures were incubated for 4 days under optimal conditions for growth. Cell viability was assessed after those 4 days by MTT assays.

3.7 MTT assays

MTT assays are commonly used to measure cell viability, by the determination of the mitochondrial activity. After four days of incubation of the cells in the different experimental conditions, the assay was started by adding the following reagents to each well: 500 µg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium bromide (MTT) and 100 µM of sodium succinate (Sigma Aldrich, Madrid, Spain). This mixture was incubated at 37ºC for two and a half hours, in order to ensure optimal reaction conditions.

Subsequently, after aspirating the culture medium, lysis solution (24V isopropanol and 1V of HCl 1M) was added (Sigma Aldrich, Madrid, Spain). Cell viability was measured with a Modulus microplate luminometer (Turner Biosystem Promega, Madrid, Spain) at a wavelength of 570 nm. Results were expressed as the percentage of cell survival relative to non-transfected control cells.

Below these lines, a complete scheme of the co-culture experiment is shown:

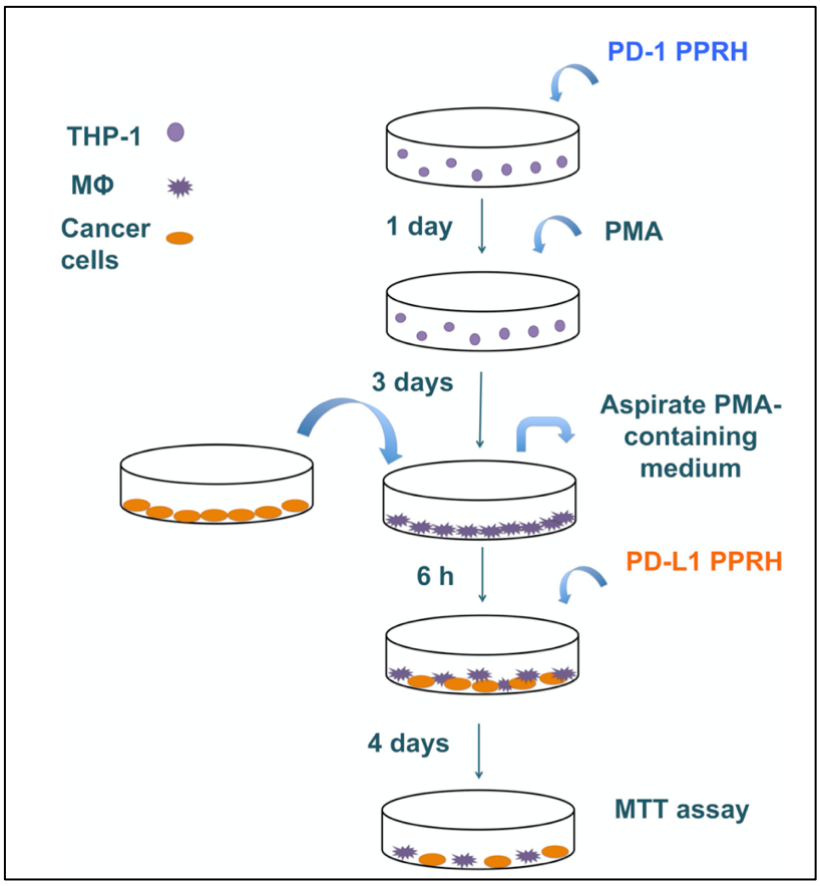

Figure 3: Coculture experiment (21).

4. RESULTS AND DISCUSSION:

4.1 Effect of PMA on THP1 cells

In order to check whether the concentration of PMA could affect the differentiation of THP1 to macrophages, we decided to test three different concentrations of the reagent: 1, 2 and 3 ng/ml.

First, we proceeded to plate a 6-well-dish with 10,000 THP-1 cells per well, so it was necessary to count the cells first, with the help of the Chamber of Neubauer. After performing the calculations, we were able to obtain the volume of cell suspension that we had to add into each well to obtain 10,000 cells, this was 34.2 µl, so we pipetted this

volume in a final volume of 1 ml of Ham's F-12 medium supplemented with 10% fetal bovine serum.

Secondly, to proceed with the differentiation it was necessary to have a 1ng/µl phorbol 12-myristate 13-acetate (PMA) solution. The stock solution was 100 ng/µl, so it was necessary to make two 1:10 dilutions, using dimethyl sulfoxide (DMSO) as a diluent, to obtain the desired solution.

Finally, the PMA solution (1 ng/µl) was added to each well. As mentioned above, 3 different concentrations were tested corresponding to 1, 2 and 3 µl of the PMA solution. The assay was performed in duplicate, as shown in the following scheme:

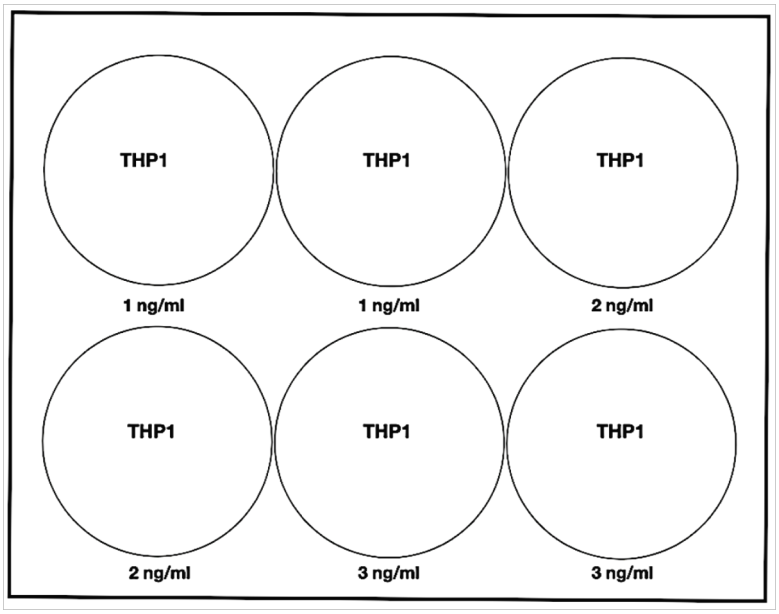

Figure 4: Scheme of PMA experiment.

The 6 well-dish was incubated for 3 days under optimal conditions for differentiation (37 ºC, 5% CO_2 controlled humified atmosphere). On the third day, we visually checked with a microscope whether the monocytes had differentiated, observing if they had adhered to the dish, and we saw that there were no significant differences among the different concentrations of PMA.

These results are in agreement with a previous study in which the differentiation of THP1 to macrophages was carried out with PMA. It was observed, after testing the three

concentrations mentioned above and determining differentiation markers, that there were no significant differences between 1 and 3 ng / ml PMA (23).

4.2 Assessment of the cross-toxicity of PPRHs

In order to evaluate the cross-toxicity effects of PPRHs, we designed the following experiment:

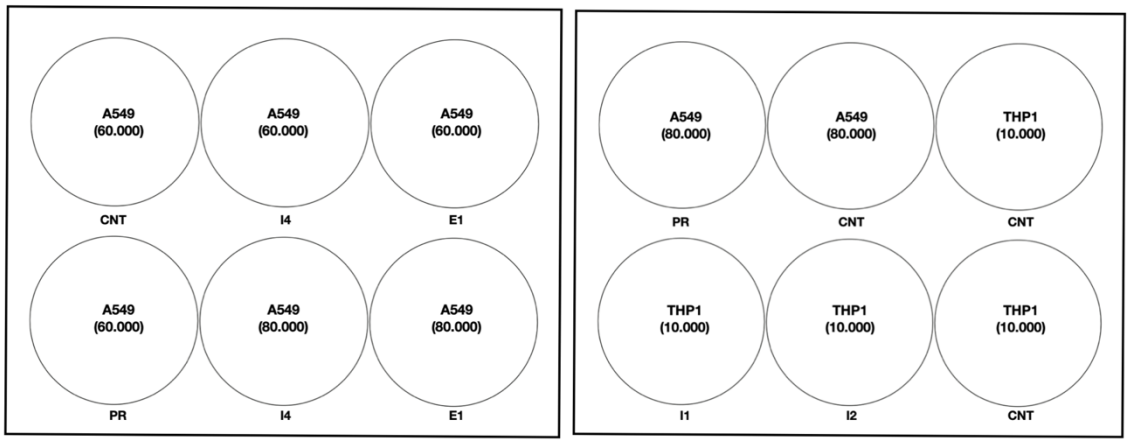

Figure 5: Scheme of cross-toxicity experiment.

As it can be seen in Figure 5, 6 well-dishes were used, in which the following conditions were tested:

- A549 and THP-1 control cells (non-transfected).
- 60,000 A549 cells transfected with anti PD1 (I4, E1 and PR).
- 80,000 A549 cells transfected with anti PD1 (I4, E1 and PR).
- 10,000 THP-1 cells transfected with anti PDL-1 (I1 and I2).

First, we proceeded to plate A549 and THP1 cells, a process for which it was necessary to count the cells using the Neubauer Chamber, following the order established during the experimental design. Two different numbers of A549 cells were used, for 60,000 it was necessary to pipet 119 µl of the cell suspension, while for 80,000 it was 158 µl. Finally, for 10,000 THP-1 cells, a volume of 34.3 µl was necessary. For the next step it was necessary to have a final volume of 800 µl in each well, so we added Ham's F-12 medium supplemented with 10% fetal bovine serum until obtaining the desired volume.

Next, we prepared the transfection mixtures for the different PPRHs against PD1 and PDL-1, by mixing 7,75 µl of DOTAP with 10 µl of the 10 µM solution of the corresponding PPRH, and 182,25 µl of serum-free medium, to reach a final volume of 200 µl. After 20 minutes at room temperature, the transfection mixtures were added to the cells.

After 4 days of transfection, we carried out MTT assays in order to determine cell viability. The results obtained after transfecting THP-1 with PPRHs against PDL-1 are shown in the table below:

THP-1 CELLS	
CONDITION	**% Viability**
CNT	100,00
+ PPRH I1	50,18
+ PPRH I2	175,55

Table 4: THP-1 cell viability, tested with PPRHs against PDL-1

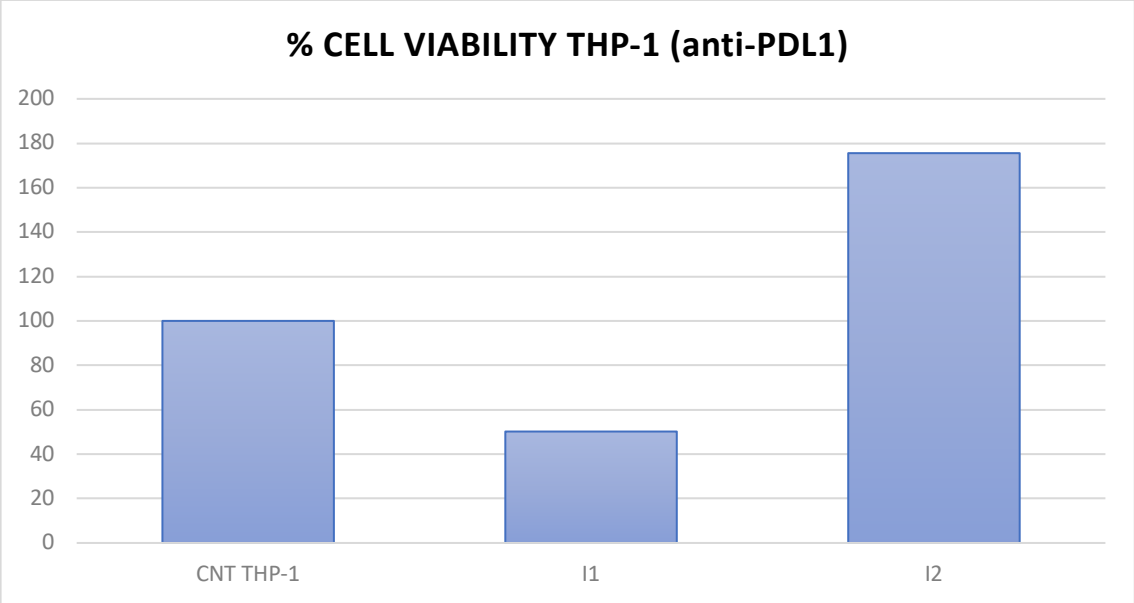

Figure 6: THP-1 cell viability in the presence of PPRHs against PDL-1

As shown in Figure 6, transfection of THP-1 cells with PPRH I1 against PDL-1 caused a reduction of almost 50% in cell viability, whereas PPRH I2 increased cell viability, which could suggest that inhibition of I2 causes an enhancement of cell growth.

Another study had tested the toxicity of PPRHs against PD1, instead of PDL-1 in THP-1 cells, in which it was observed that the transfection of PPRHs against PD1 in this cell line did not cause significant effects on cell viability (21).

The following table shows the results obtained after performing the MTT assay on the A549 transfected with the PPRHs against PD1:

A549 CELLS		
	CONDITION	**% Viability**
Control	*CNT*	100,00
60.000	*+ PPRH I4*	49,73
	+ PPRH E1	63,14
	+ PPRH PR	58,75
80.000	*+ PPRH I4*	69,02
	+ PPRH E1	85,97
	+ PPRH PR	82,64

Table 5: A549 Cell viability, tested with PPRHs against PD1

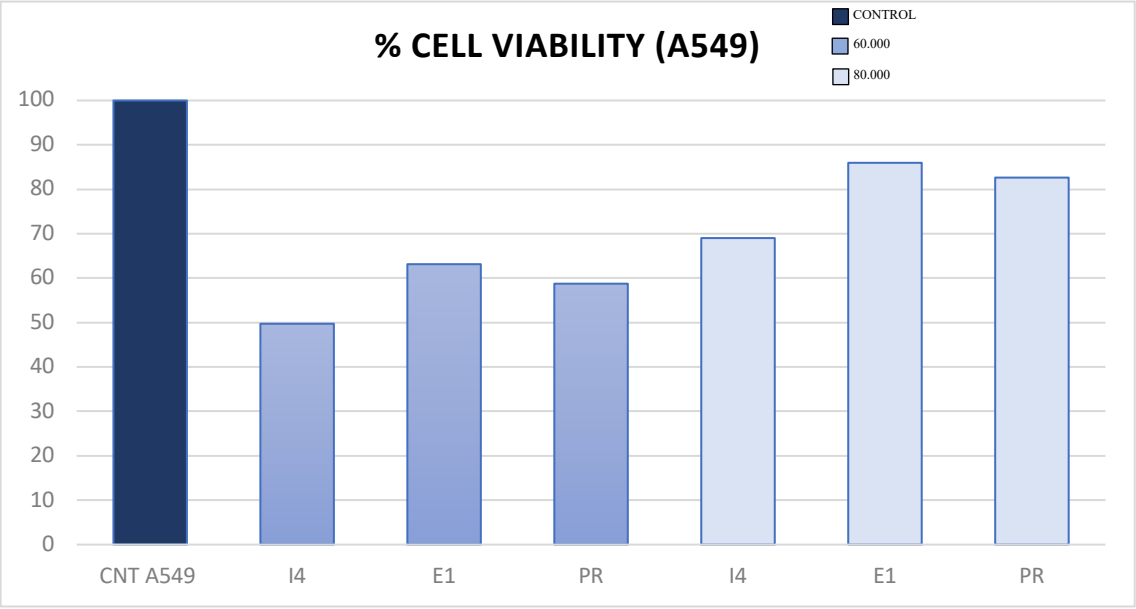

Figure 7: A549 cell viability compared to the control cells in the presence of PPRHs against PD1

We also evaluated the cross-toxicity effects of the PPRHs against PD1 in A549 cells. In this case, all PPRHs decreased cell viability, being I4 the responsible for the most pronounced decrease, both with 60,000 and 80,000 cells.

In prostate cancer cells, no cytotoxic effects were observed when transfecting these cells with PPRHs against PD1 (21), thus the difference in cell viability could be due to an increase of sensitivity of A549 to PPRH transfection.

On the other hand, toxicity of PPRHs against PDL-1 in prostate cancer cells was also determined. Both PPRHs against I1 and I2 caused up to a 65% decrease in cell viability (21).

The reason to work with two different numbers of cells, 60.000 and 80.000, was to see if cell density could affect PPRH toxicity, in accordance with the study referred above (21). Bener et al. performed co-culture experiments with 60,000 breast cancer cells (23). In our case, we finally decided to use 60,000 A549 cells in the co-culture experiments, due to the high rate of cell division for this cell line.

4.3 Evaluation of the effect of PPRHs on cell viability in co-culture experiment:

To evaluate the combined effect of PPRHs on cell viability, we designed a co-culture experiment with A549 and THP1 cells, which can be seen in the following scheme:

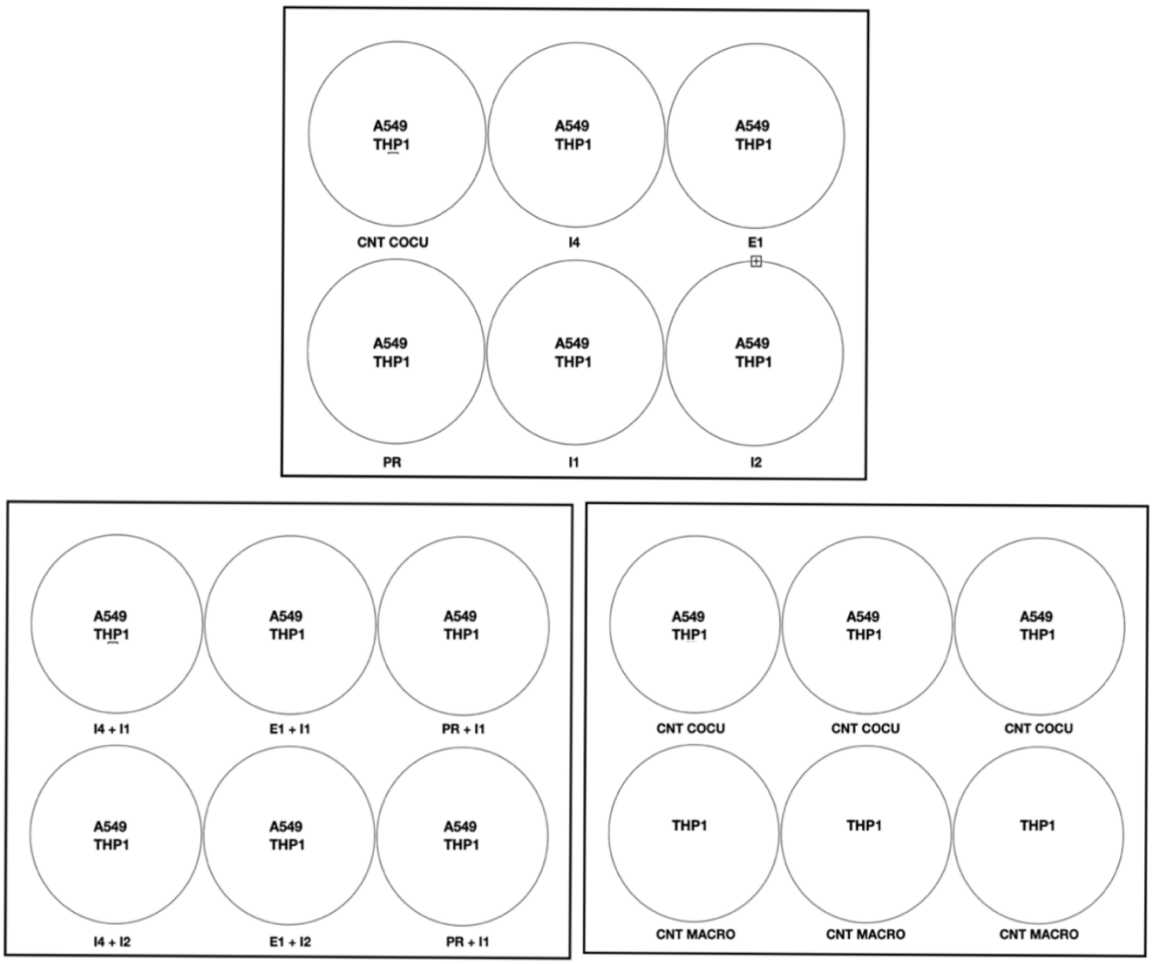

Figure 8: Scheme of the co-culture experiment.

The experiment was carried out in three 6-well-dishes, in which we tested the following conditions:

- Coculture of A549 and THP1 without transfection: CNT COCU.
- THP1 transfected with PPRHs against PD1 and differentiated: CNT MACRO.
- Transfection of individual PPRHs in co-cultures: I4, E1, PR, I1 and I2.
- Combination of PPRHs against PD1 and against PDL-1 in co-cultures: I4+I1, I4+I2, E1+I1, E1+I2, PR+I1 and PR+I2.

In order to carry out this experiment, first 10.000 THP1 were plated in each well, a process for which it was necessary to perform the counting through the Neubauer chamber. We pipetted 26.5 µl of cell suspension for 10,000 cells. For the subsequent

transfection, a final volume equal to 800 µl was necessary, so we also added 773.5 µl of Ham's F-12 medium supplemented with 10% fetal bovine serum to each well.

Next, we proceeded to prepare 200 µl of transfection mixtures of the PPRHs against PD1 (I4, E1, PR), by mixing 182.25 µl of serum-free medium, 7.75 µl of DOTAP and 10 µl of the 10 µM solution of the corresponding PPRH. After 20 minutes at room temperature, the mixtures were added to the corresponding wells.

Twenty-four hours after transfection, we started differentiating THP1 into macrophages by adding 2 ng/ml of PMA in each well and incubating for 3 days. On the third day, we visually checked that THP1 cells have been correctly differentiated to macrophages and aspirated the medium to start the co-culture.

We plated 60,000 A549 cells in all wells except those marked as macrophage control. This number of cells corresponded to 59.5 µl of cell suspension, in addition to 740.5 µl of Ham's F-12 medium supplemented with 10% fetal bovine serum. Before transfection, we incubated the co-culture for 6 hours.

Finally, we prepared the transfection mixtures of the PPRHs against PDL-1 (I1 and I2), as previously described. After 20 minutes of incubation at room temperature, the 200 µl of transfection mixture were added into each well.

Co-cultures were incubated for 4 days. On the fourth day, we carried out the MTT analysis. Cell viability was determined taking into account the absorbance of macrophages alone, which was subtracted from the total absorbance, in order to obtain the value for A549 cells. Cell viability was expressed as the percentage of living transfected A549 cells compared to non-transfected A549 cells. The results for the co-culture experiments are shown below:

A549 CELL VIABILITY	
CONDITION	**% Viability**
CNT	100,00
+ *PPRH I4*	100,00
+ *PPRH E1*	95,84
+ *PPRH PR*	81,93
+ *PPRH I1*	59,87
+ *PPRH I2*	42,32

Table 6: Cell viability, testing PPRHs individually.

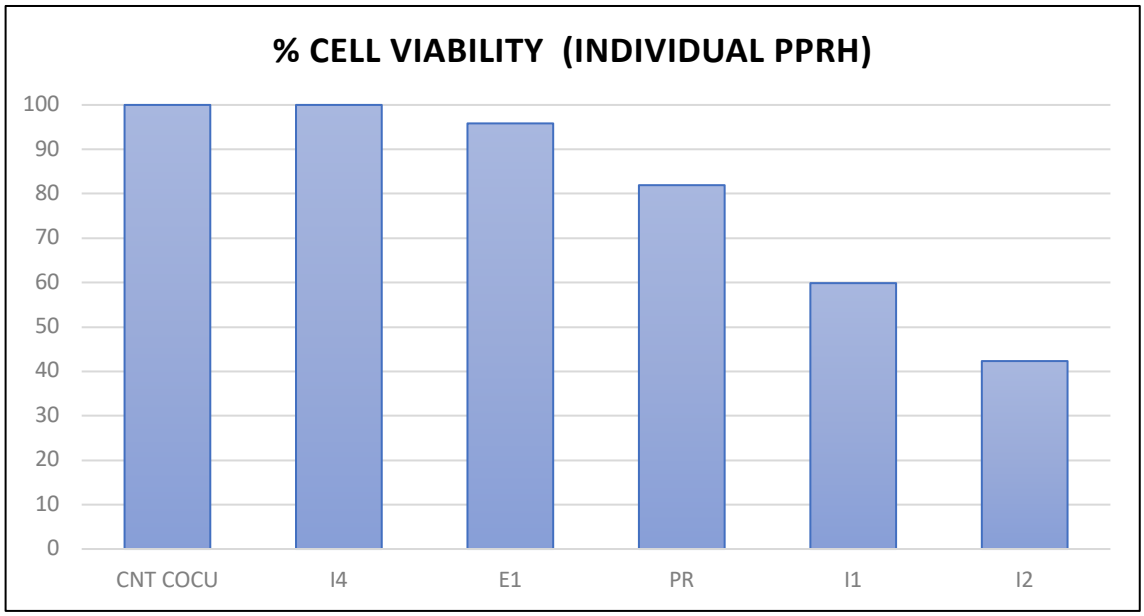

Figure 9: % of cell viability compared to the control cells when testing individual PPRH.

As can be seen in the previous figure, PPRHs against PD1 tested individually, did not cause a significant decrease in A549 cell viability, while those directed against PDL-1 affected cell survival up to 60%.

In a similar study, Medina Enríquez et al. reported, as in our experiment, that PPRHs directed against PDL-1 (I1 and I2) caused a greater decrease of prostate cancer cell viability, up to 66%, while those directed against PD1 did not reach 40% of decrease (21).

To check if the combination of PPRHs against PD1 and PDL-1 could lead to an enhancement in tumor cell death, we transfected both THP1 and A549 cells in the same co-culture with all possible combinations. The cell viability results are shown below.

A549 CELL VIABILITY	
CONDITION	**% Viability**
CNT COCU	100,00
+ PPRH I4/I1	50,06
+ PPRH E1/I1	47,84
+ PPRH PR/I1	54,11
+ PPRH I4/I2	41,61
+ PPRH E1/I2	44,35
+ PPRH PR/I2	38,48

Table 7: Cell viability, testing PPRHs combined.

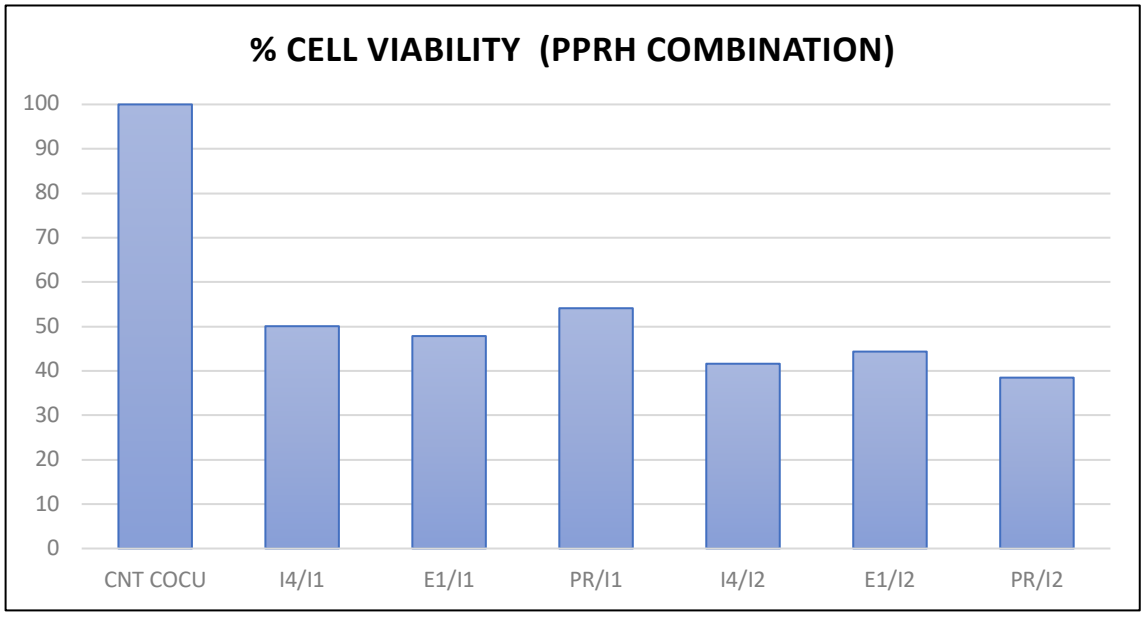

Figure 10: % of cell viability compared to the control cells when testing PPRHs in combination.

The above graph shows the results of cell viability, using all possible combinations of PPRHs against PD1/PDL-1. It should be noted that all combinations reduced cell viability by at least 55%, with the steepest decrease corresponding to the PR/I2 combination, reason why we could say that the inhibition of both increases cell death.

The combination of PPRHs against PD1 and PDL-1 in co-culture experiments with prostate, pancreas, melanoma and breast tumor cells was previously assayed. The most effective combinations were PR/I1 in prostate cancer, E1/I1 in melanoma and pancreatic cancer and E1/I2 in breast cancer. These results could indicate that cell response to PPRHs inhibition could depend on the tumor type (21).

5. CONCLUSIONS:

- The usage of the PPRHs technology against the PD1 and PDL-1 pathway markedly reduces the viability of lung adenocarcinoma cells.

- Transfection of A549 cells with PPRHs against PD1 could represent a new approach as anticancer treatment in lung cancer.

- Silencing of both PD1 and PDL-1 by PPRHs enhances tumor cell death.

- PPRHs against PD1 / PDL-1 could be a new research strategy in the field of immunotherapy against lung cancer.

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