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Identification of treatments targeting PML-expressing breast cancers

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INTRODUCTION

Breast cancer is a malignant tumor that starts with the transformation of breast epithelia cells. A malignant tumor is a group of cancer cells that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can suffer it too. *(Gupta, G.P. & Massague, 2006)*

Moreover, breast cancer is a heterogeneous disease with multiple tumor subtypes (*Sorlie, T., et al., 2001).* These subtypes vary in tumor gene expression and phenotype, and are most commonly grouped into four major subtypes on the basis of immunohistochemical staining: (1) luminal A: Hormone Receptor (HR) positive (ER (Estrogen Receptor) or PR (Progesterone Receptor) positive), (2) luminal B: (HR positive + HER2 positive or HR positive + HER2 negative with high Ki67), (3) HER2-overexpressing and (4) triple negative (or basal-like). *(Barnard, ME. et al., 2015; Ha, R. et al.2015).*

Those with hormone receptors depend on the hormones to grow, therefore, drugs can be applied to block the receptors and stop tumor growth, consequently having a better prognosis. Triple-negative breast cancer is a subtype of breast cancer that is more aggressive and has distinct disease etiology. Due to the lack of an effective targeted medicine (being HR and HER2 negative), treatment options for triple-negative breast cancer are few and recurrence rates are high. (*Kuo, WH. et al., 2012).*

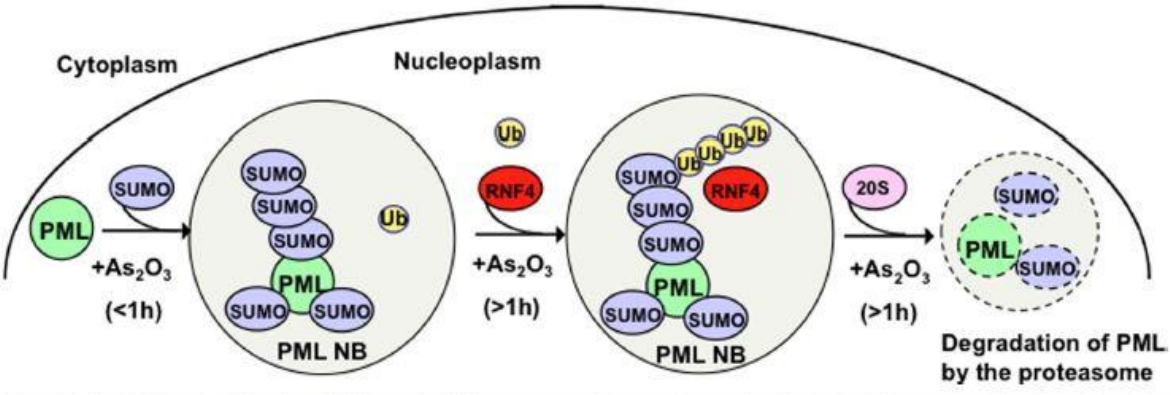
PML (Promyelocytic Leukemia protein) was first discovered as a part of a fusion oncoprotein and the current view is that it is a key regulator underlying leukemia and other cancers. *(Martin-Martin et al., 2013)*.

PML is a member of the TRIM/RBCC family of proteins, a group that contains a variety of ubiquitin ligases that generate subcellular structures through autoassembly *(Reymond et al., 2001; Meroni and Diez-Roux, 2005).* Among other structures, PML has an amino-terminal RING finger that directly binds the SUMO E2 ligase UBC9 *(Duprez et al., 1999)*, therefore, PML can be sumoylated and build the characteristic nuclear bodies. PML nuclear bodies are highly regulated structures, which work as scaffolds for different proteins, and therefore, they control a wide array of functions in the cell. (*Carracedo, A., Ito, K. & Pandolfi, P.P., 2011).* In triple negative breast cancer cells, PML levels are upregulated and nuclear bodies play a pro-survival role. (*Carracedo, A., et al. 2012)*. Considering this, we have postulated that PML is a therapeutic target in triple negative breast cancers and reduction of PML expression may be an approach to treat these breast cancers.

Arsenic trioxide (ATO) binds specifically to PML through oxidation-triggered disulfide bond formation and induces sumoylation of K160, followed by proteasome dependent degradation. *(Lallemand-Breitenbach, V et al., 2010).*

This ubiquitination is RNF4 (RING finger protein 4, an E3 ligase)-mediated, which recognizes the ATO-induced sumoylation and incorporates ubiquitin molecules for proteasome degradation *(Tatham, MH. et al., 2008)* shown in **Picture 1**.

Picture 1. RNF mediated degradation of PML by the proteasome, induced by ATO



PML can be regulated at multiple levels: transcriptionally through different transcription factors, post-translationally, as the sumoylation described above and post-transcriptionally, due to the alternative splicing, resulting in different isoforms that have different molecular weight. (*Bernardi and Pandolfi, 2007*). Nevertheless, it has been shown that Arsenic trioxide binds to all isoforms and therefore, induce their degradation equally.

In every drug treatment, the main goal is to minimize the dose to avoid side effects in the patient. In this case, to treat triple negative breast cancer, the aim is to combine a less harmful ATO dose with already approved chemotherapy drugs. In this way, combining both therapies in much less dose than when applied individually, we look for a synergystic effect (their combined effect is greater than the sum of their individual effects).

FDA approved chemotherapy drugs that are used in this project are Cisplatin, Doxorubicin, Paclitaxel and Cyclophosphamide:

- Cisplatin: it crosslinks with the purine bases on DNA and interfere with DNA repair causing DNA damage. Due to the incapacity of repair, cells enter in apoptosis.
- Doxorubicin: it intercalates into DNA and disrupts topoisomerase-II mediated DNA repair / generates free radicals damaging membranes, protein and DNA.
- Paclitaxel: interferes with the normal breakdown of microtubules during cell division.
- Cyclophosphamide: interferes with DNA replication by forming intrastrand and interstrand DNA crosslinks.

The main aim of this project is to find a suitable ATO concentration to combine with already approved chemotherapeutic agents to find that synergistic effect in triple negative breast cancer MDA-MB 231 cell line, as a new strategy to treat the disease. Additionally, the Carracedo laboratory has generated a CRISPR technology mediated modified osteosarcoma cell line. These cells present a fusion of YFP to exon 1 of PML in frame. Therefore, we can monitor equal PML and YFP-PML degradation upon ATO treatment to establish the potential of this tool for future drug discovery screening, looking for PML regulators.

MATERIALS AND METHODS

Cell lines

Two different cell lines were used along the project:

- MDA-MB-231 cell line: A human triple negative breast cancer cell line.
    - U2OS cell line: Human bone osteosarcoma epithelial cell line. We used this cell lines due to the inability to generate CRISPR-targeted cells in MDA-MB 231 due to a problem with the promotor when using CRISPR. The modification in U2OS cell line could be done successfully. We thus generated U2OS YFP-PML cells, with YFP-PML expression from the endogenous PML locus.

Tissue culture

Thawing cells

All used cell lines were stored at -196ºC in 10% DMSO (dimethyl sulfoxide) and 90% fetal bovine serum. They were thawed in 37ºC water bath and transferred into a p100 (100 mm petri dish) with 15 ml of previously warmed complete media (DMEM + 10% fetal bovine serum + 1% penicillin/streptomycin). The plate was stored at 37ºC overnight with 5% CO_2 in incubator and the media was changed the next day to avoid longer DMSO exposure, because it can affect cell growth.

Cell seeding

On one hand, cells from MDA-MB 231 cell lines were seeded in 96-well plates (for growth curve analysis) and 6-well plates (for PML degradation analysis). For this purpose, the cells grew for 2-3 days and after detaching with trypsin, were counted in a Neubauer chamber.

- To choose the appropriate ATO and cell concentrations for the therapeutic combination experiments, 4 different cell concentrations were seeded: 1000, 500, 250 and 125 cells/well and 12 wells per concentration in the 96-well plates (200 µl/well).
- To monitor PML degradation, 100000 cells/well were seeded in 6-well plates (2 ml/well).

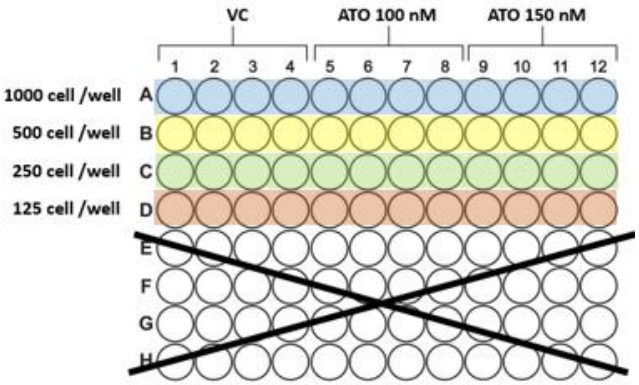
On the other hand, cells from U2OS and U2OS-YFP-PML cell lines were seeded in 24-well plates to monitor PML degradation. 50000 cells/well were seeded.

Drug treatments

The three cell lines were treated with ATO (in different concentrations) to see the degradation of PML and cell death. For that, two different stocks were prepared to reach the desired concentrations: stock I in NaOH 1N and stock II in PBS. Apart from that, the MDA-MB 231 cell line was treated with combined therapy: ATO + chemotherapy with the aim of determining synergistic effects (their combined effect is greater than the sum of their individual effects) when applying both therapies.

MDA-MB 231 cell line:

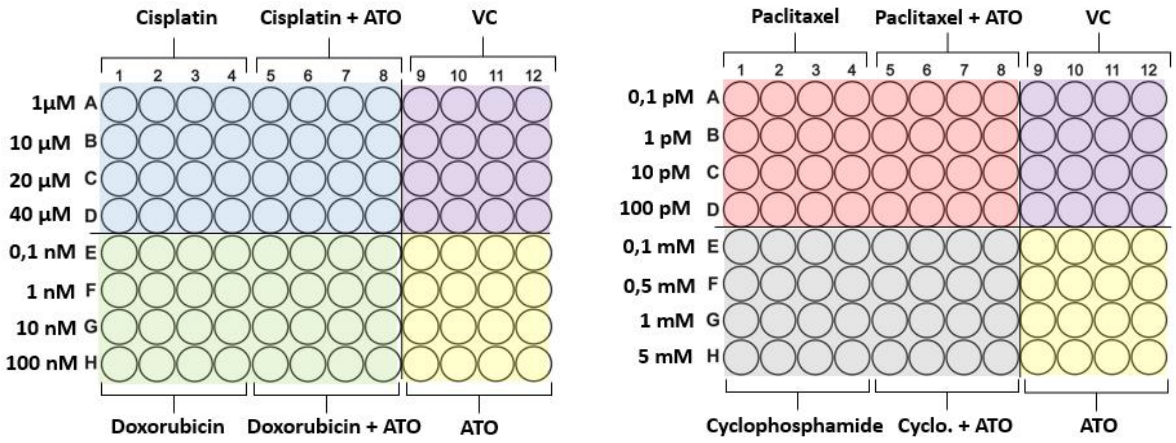
- ATO treatments were done in 96-well plates and 6-well plates 24h after seeding day. Different cell and ATO concentrations were used to choose the most representative cell and ATO concentrations and monitor PML degradation by ATO:
    - Cells: in 96-well plates 1000, 500, 250 and 125 cells/well and in 6-well plates 100000 cells/well, as described before.
    - Treatments (50 µl): VC (no treatment: only media, control), 100 nM ATO and 150 nM ATO.



- Combined treatments were done in 96-well plates with 125 cells/well (total cell + treatment volume = 230 µl) 24h after seeding day. Different chemotherapeutic drugs (20 µl) in different concentrations.
    - Cisplatin (1µM, 10µM, 20µM and 40µM) in DMSO (20µl)
        - + ATO 150 nM (10 µl) or + media (10 µl)
    - Doxorubicin (0,1 nM , 1 nM, 10 nM and 100 nM) in H_2O (20 µl)
        - + ATO 150 nM (10 µl) or + media (10 µl)

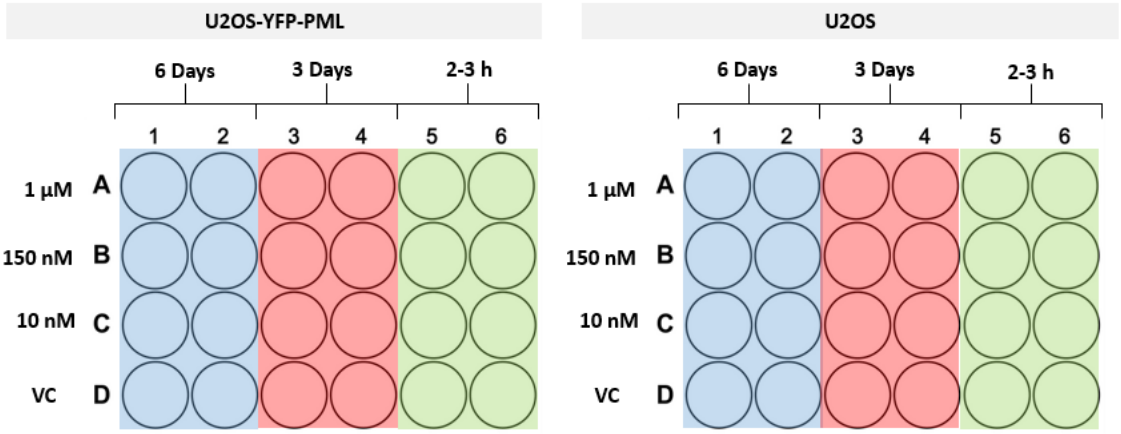
- Cyclophosphamide (0,1 mM, 0,5 mM 1 mM and 5 mM) in H_2O (20 µl)
  - + ATO 150 nM (10 µl) or + media (10 µl)
- Paclitaxel (0,1 pM, 1 pM, 10 pM and 100 pM) in DMSO (20 µl)
  - + ATO 150 nM (10 µl) or + media (10 µl)

- VCs (controls): no treatment, only media and DMSO or H_2O (depending on drug´s solvent in combined treatments) were added.



U2OS and U2OS-YFP-PML cell lines:

- ATO treatments were done in 24-well plates with 50000 cells/well for the following duration: 2-3 h, 72 h and 144 h after seeding day, applying 10 nM, 150 nM and 1 µM ATO concentrations.



<u>Freezing plates</u>

In MDA-MB 231 cells, the 6-well plates for protein quantification were frozen at days 3 and 6 after the treatment with ATO to monitor the degradation of PML.

All the wells were washed with PBS and after discarding it, the plates were frozen in liquid nitrogen and stored at -80 ºC until all the experiments were finished.

In U2OS cells, the 24-well plates were frozen after the whole experiment following the same procedure as with 6-well plates.

Crystal violet staining (in MDA-MB 231 cells)

The crystal violet staining measures total attached cell number. Therefore, measuring cell number along time, growth curves were done and this can reflect changes in proliferation and survival.

Fixations of the 96-well plates were done at day 3 and day 6 in each experiment with 10% formalin solution (100 µl/well). All fixed plates were stored at 4ºC until the complete experiment was finished.

Then, after discarding the formalin and washing the wells with PBS, cells were stained with crystal violet for 40 minutes (200 µl/well), washed with dH_2O and dry overnight.

Finally, stained cells were resuspended in acetic acid (100 µl/well) and the absorbance was quantified at 595 nm wavelength.

Protein extraction and quantification

The frozen 6-well plates that were stored at -80ºC were thawed and 75µl of RIPA (lysis buffer: 50 mM Tris-HCl (pH 7,5) (Melford), 150 mM NaCl, 1 mM EDTA (Amresco), 1% NP-40, 1% Sodium deoxycholate, 0,1% Sodium dodecyl sulfate (SDS), 1 pill complete protease inhibitors (Roche), 1 mM sodium fluoride, 1mM sodium orthovanadate and 1 mM β-glycerophosphate) were added in each well to lyse the cells (2 minutes). After that, the lysed cells were collected in eppendorf tubes using a scrapper and a pipette and left in a roller for 30 minutes at 4ºC. Then, the tubes were centrifuged 14000g, 10 minutes at 4ºC and the supernatant was collected in new tubes, avoiding the pellet (membranes, nucleic acids…).

To quantify protein concentration extracts, BCA method was used: a standard curve was performed with known BSA concentrations and 200µl BCA reactive. The extracted proteins (1µl + 9µl H_2O) with the same reactive agent were compared to this pattern. The plate was read at 562 nm after the incubation for 30 minutes at 37ºC. All the samples were measured in duplicate for consistency.

Next, calculations were done to normalize protein concentration among samples by adding H_2O. Laemmli reactive 5X was added proportional to the volume of the samples. Finally, all the samples were boiled at 95ºC for 5 minutes and stored at -20ºC.

Western blot

Western blot is a technique used to detect specific proteins. A gel electrophoresis is performed to separate the proteins depending on their size/charge ratio and the proteins are then transferred by voltage to a nitrocellulose membrane. Then, membranes are incubated with specific antibodies and the signal is developed adding ECL (250 mM Luminol, 90 mM coumaric acid, 1 M Tris (pH 1,5) and H_2O) to membranes.

In this case, PML was detected and the signal was normalized to β-Actin, assuming that β-Actin expression doesn´t change in different cell lines under ATO treatment) to monitor PML degradation over time.

Stored protein preparations were boiled at 95ºC for 5 minutes and 10 µl were loaded in a precast gel (Nupage gradient gel 4-12%, Life Technologies) with a lane dedicated to the molecular weight marker (7 µl). A voltage of 100-200 V was applied to separate the proteins in the gel electrophoresis.

After that, the proteins were transferred into a nitrocellulose membrane with wet transfer system. A voltage of 100 V was applied for 1,5 h. Next, the membrane was blocked with milk 5% in TBST (TBS (tris buffered saline) + Tween 0,1%) for 1h approximately and then, incubated with the primary antibodies overnight (anti-PML 1:1000 and anti-β-Actin (control) 1:2000). After that, the primary antibodies were removed and the membranes were washed with TBST and incubated for 1h at room temperature with the corresponding secondary antibodies (anti-rabbit for PML detection and anti-mouse for β-Actin, respectively, in 1:4000 proportion). The secondaries also were removed and the membranes were washed with TBST before adding ECL to develop.

When doing a western blot with proteins extracted from U2OS and U2OS-YFP-PML cell lines after treatment, also YFP signal was detected using anti-GFP primary antibody and its respective anti-rabbit secondary antibody. Unspecific bands were detected when developing and therefore, an alternative anti-GFP primary antibody was used to detect the specific signal. This time, the secondary antibody used was anti-mouse secondary antibody.

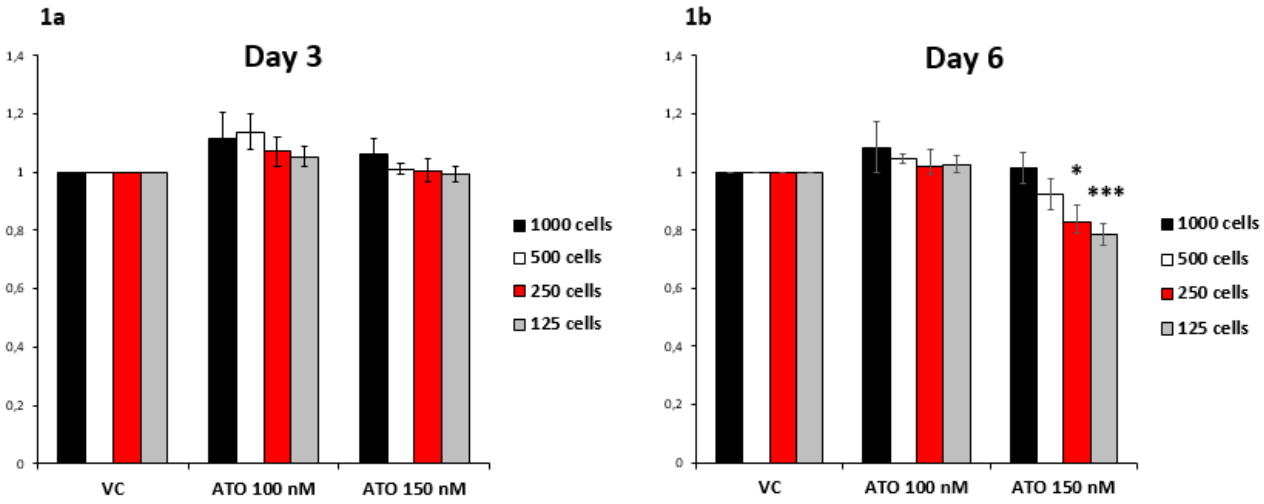
Stripping anti-PML antibody

In the extracted proteins from U2OS-YFP cell line we also wanted to see YFP signal and the secondary antibody for anti-GFP antibody is anti-rabbit (the same as the anti-PML´s), therefore, membranes were stripped using NaOH 0.4 N solution for 5 minutes. After that, the membranes were washed with TBST, blocked with milk 5% and incubated again with the corresponding primary and secondary antibodies. The stripping was repeated with other stripping buffer (Restore™ PLUS Western Blot Stripping Buffer, Thermo Scientific) because the interaction between PML and the anti-

PML antibody is very strong and high pH induced by NaOH was not enough to break that interaction. However, the second stripping buffer did not result in antibody removal either.

RESULTS

Choosing cell and ATO concentrations in MDA-MB 231 cell line (Figure 1a and 1b)

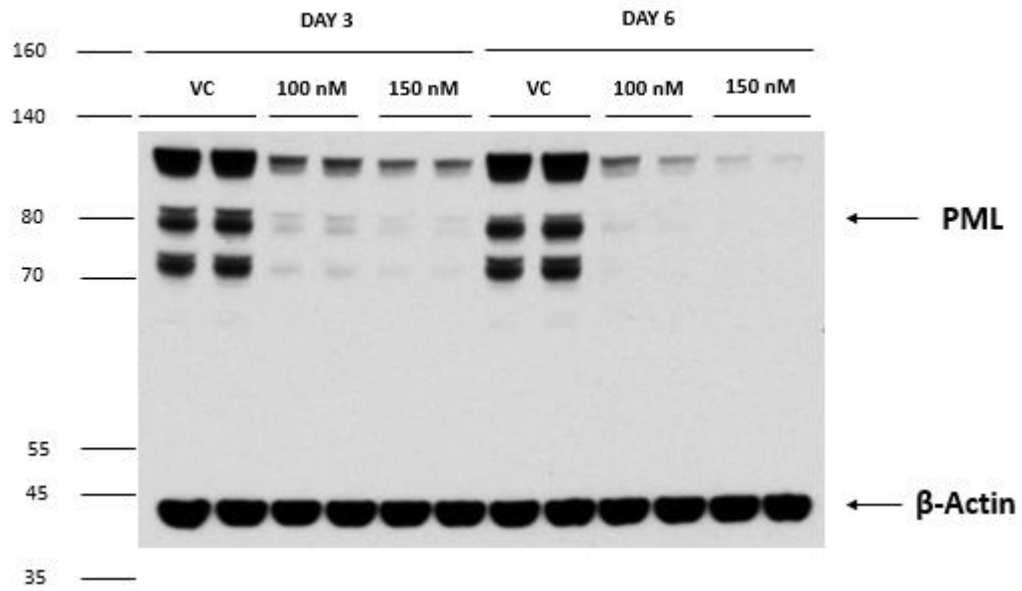


Different cell concentrations were cultured with various ATO concentrations in order to define the best settings for combined therapy analysis.

As it can be observed in **Figure 1a** and **1b**, relative cell number after treatment vs incubation with vehicle is presented. The plates were fixed 3 days and 6 days after treatment, but we only observed changes in cell viability at day 6 (**Figure 1b**). In this graph, 4 different cell concentrations are plotted. Of note, the highest cell concentrations (1000 and 500 cells/well), ATO treatment did not induce a differential response to that observed with 250 and 125 cells. In addition, a significant cell number decrease was not observed with 100 nM ATO treatment and neither 3 days after treatment.

At day 6 after treatment, the relative cell number decreased significantly (around 20%) in 250 and 125 cells/well concentrations, being the p values 0,030285 (*) and 0,000355 (***) respectively (**Figure 1b**). Therefore, the chosen ATO concentration for the combined treatment with chemotherapy was 150 nM ATO and a cell concentration of 125 cell/well.

Western blot of ATO treatment in MDA-MB 231 cell line (Figure 2)

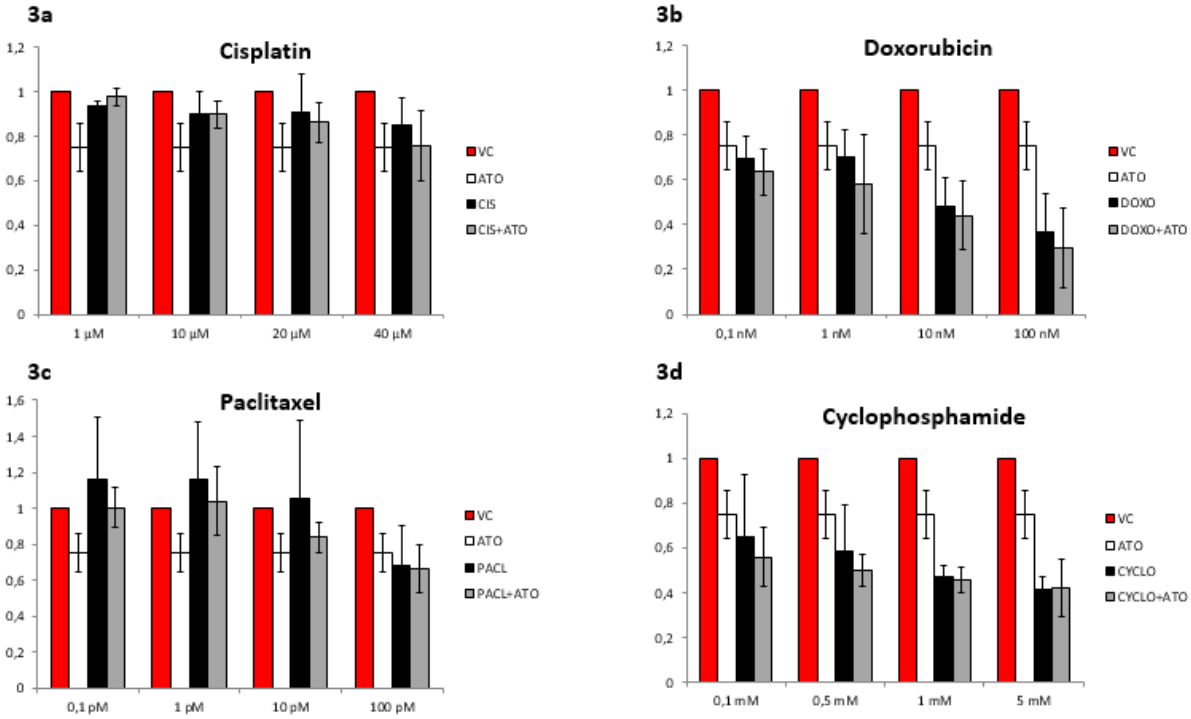


Western blot was performed with proteins extracts from MDA-MB 231 cells to determine PML and β-Actin (as a control) protein expression and monitor PML degradation by ATO in different concentrations. As it can be observed in **Figure 2**, PML immunoreactive bands were observed with sizes ranging 70-120 kDa due to its different isoforms. β-Actin bands appear slightly below 45 kDa, as expected, because its molecular weight is 43 kDa.

PML degradation can be nicely observed in cells under treatment (either 100 nM or 150 nM ATO) compared with the controls (VC, not treated). As expected, increasing concentrations of ATO (100 nM → 150 nM) and longer time points, led to a progressive decrease in PML levels.

β-Actin levels had exhibited no difference associated to ATO or days in culture.

Results of the combined treatment in MDA-MB 231 cell line (Figure 3a, 3b, 3c and 3d)



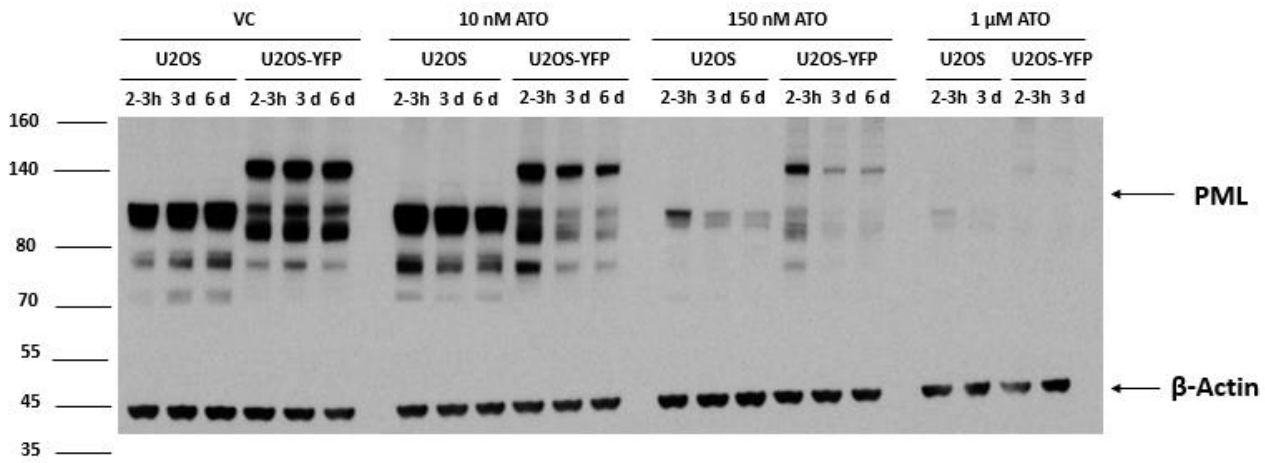
A combinatory treatment was performed in 96-well plates where MDA-MB 231 cells were seeded. In these experiments, a synergistic effect was studied when applying a combination of 150 nM ATO + one of the four chemotherapeutic drug treatments. Four different graphs were plotted reflecting relative cell number, the combinatorial regime and the concentration of the chemotherapeutic agent.

As it can be observed in all graphs, ATO treatment induced a 20 % decrease in relative cell number compared with the controls (VC). Each drug had a dose-dependent effect in cell number decrease (except for Cisplatin, **Figure 3a**). In the case of Doxorubicin and Cyclophosphamide, even with the lowest drug concentration applied, a decrease in cell number of about 30% could be observed, as shown in **Figure 3b** and **3c**. Increasing drug concentrations (100 nM Doxorubicin and 5 mM Cyclophosphamide), cell number was further decreased by 70% and 60%, respectively.

On the other hand, the range of Paclitaxel and Cisplatin doses employed were below the IC50. In the case of Paclitaxel, we observed a moderate reduction in cell number only with the highest dose (100 pM) (about 30%) (**Figure 3d)**. In the case of Cisplatin, the cell decrease was more or less consistent with all applied drug concentrations (about 10%) **(Figure 3a)**.

We did not observe synergistic effects in any of the combinatorial treatments.

Western blot of ATO treatment in U2OS and U2OS-YFP-PML cells (Figure 4)



In this Western blot, PML and YFP-PML degradation were compared under ATO treatment to check whether YFP fusion might influence protein degradation. In the modified U2OS cell line with YFP fused to PML, a shift in the molecular weight of PML isoforms was expected due to the molecular weight of YFP (30 kDa), which fused to PML would lead to larger PML isoforms. This effect can be nicely appreciated in **Figure 4**, where all isoforms of U2OS-YFP-PML cell line are 30 kDa above the original PML bands. This blot also showed that the YFP-PML modification in the U2OS cell line was homozygous, meaning that the fusion copy was inserted in both alleles, because being heterozygous, those lower bands around 70 kDa should have also appeared in U2OS-YFP-PML cells.

Regarding PML degradation, ATO treatment affected YFP-PML degradation to a similar extent of the endogenous. Even with a low concentration of ATO (10 nM) a slight degradation could be observed in both cell lines and with a high concentration of ATO (1 µM) nearly no PML or YFP-PML was detected **(Figure 4)**. β-Actin levels remained unaffected in both cell lines after ATO treatment, as expected (control).

To conclude, we can confirm that YFP fusion does not affect in PML degradation.

DISCUSSION

With this project, we want to explore an *in vitro* method to identify drug combinations with therapeutic potential in breast cancer. As mentioned before, triple-negative breast cancer is a subtype of breast cancer with poor prognosis and resistance to conventional therapy. Due to the lack of an effective targeted medicine, treatment options for triple-negative breast cancer are few and recurrence rates are high. (*Wen-Hung Kuo et al. PLoS One. 2012; 7(9): e45831 Published online 2012 Sep 25).*

As reported before, we hypothesize that PML is a therapeutic target in triple negative breast cancers and reduction of PML expression may be an approach to treat this tumor type. In this project, we show that 150 nM ATO reduces in 20 % the cell population at day 6 in MDA-MB 231 triple negative breast cancer cells, and therefore, a combination therapy could offer the advantage to obtain synergistic effects.

In the evaluation of combination of ATO with first line chemotherapy, we did not get any synergistic effect in our combined therapy (shown in **Figures 3a, 3b, 3c** and **3d**). Our future work is focused on screening for small molecules that can synergize with PML inhibition and identify combinations with therapeutic potential. In this project, we show a method to monitor PML degradation by fluorescence, using modified U2OS cells. As we have presented in **Figure 4**, YFP fusion does not affect ATO-induced PML degradation and consequently, those cells can be used to monitor PML degradation by fluorescence detection and test different drugs searching for synergistic effects. Furthermore, the fact that we have used small plates (96-well plates) to make the experiments, allows us to screen a high number of compounds simultaneously in cell lines and transferring those experiments to a smaller plate (384-well plate), would lead to high throughput monitoring, saving a lot of time.

FUTURE PERSPECTIVES

The Carracedo laboratory has already spoken with a pharmaceutical initiative, which would offer a small molecule library to test with this method.

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