Combination Treatment of Retinoic Acid Plus Focal Adhesion Kinase Inhibitor Prevents Tumor Growth and Breast Cancer Cells Metastasis

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Research Article

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Abstract

All-trans retinoic acid (RA) is the primary metabolite of vitamin A and controls the development and homeostasis of organisms and tissues. RA and its natural and synthetic derivatives, known as retinoids, are promising agents in treating and chemoprevention different neoplasias, including breast cancer (BC). Focal adhesion kinase (FAK) is a crucial regulator of cell migration, and its overexpression is associated with the metastatic behavior of tumors. Thus, pharmaceutical FAK inhibitors (FAKi) have been developed to counter its action. In this work, we hypothesize that RA plus FAKi (RA+FAKi) approach could improve inhibition of tumor progression. By *in silico* analysis, we confirmed RARA, SRC, and PTK2 (encoding RAR α , Src, and FAK, respectively) overexpression in all breast cells tested. In metastatic BC cells, we reveal that genes encoding proteins that FAK directly or indirectly modulates are deregulated compared to normal cells. We showed a different pattern of genes up/down-regulated between RA-resistant and RA-sensitive BC cells. In addition, we demonstrated that both RA-resistant BC cells (MDA-MB-231 and MDA-MB-468) display the same behavior after RA treatment, modulating the expression of genes involved in Src-FAK signaling. Furthermore, we demonstrated that although RA and FAKi administered separately decrease viability, adhesion, and migration in mammary adenocarcinoma LM3 cells, their combination exerts a higher effect. We also evidenced that RA effects are extrapolated to other cancer cells, including the human cervical carcinoma cells HeLa. In an orthotopic assay of LM3 tumor growth, RA and FAKi administered separately reduce tumor growth; however, the combined treatment induces the more potent inhibition increasing mice survival. Moreover, in an experimental metastatic assay, RA significantly reduces metastatic lung dissemination of LM3 cells. Overall, these results indicate that RA resistance would reflect deregulation of most RA-target genes, including genes encoding components of the Src-FAK pathway. Our study demonstrates that RA plays an essential role in disrupting BC tumor growth and metastatic dissemination *in vitro* and *in vivo* by controlling FAK expression and localization. RA plus FAKi exacerbated these effects suggesting that the sensibility to RA therapies could be increased with FAKi coadministration in BC tumors.

Introduction

All-trans-retinoic acid (RA) is a biologically active derivate of vitamin A with an established role in cell growth, differentiation, and apoptosis [1, 2]. RA exerts its effects through nuclear receptors called retinoic acid receptors (RARs: RAR α , RAR β , and RAR γ), which form heterodimers with retinoid-X-receptors (RXR) to control the transcription of target genes. The transcription of more than 3000 human genes is associated with RA modulation due to retinoic acid response elements (RARE) in their promoter regions [3, 1]. Inactivated RAR-RXR heterodimers form complexes with corepressors to block transcription by interfering with the access to chromatin [4]. In contrast, RA binding released corepressors and thereby, coactivators take their place to allow access to chromatin [5, 6]. RA can also function via non-genomic pathway. When RA binds to a fraction of the RARs pool present in the plasma membrane, they are rapidly and efficiently activated, resulting in the induction of multiple kinases and scaffold proteins involved in several signal transduction leading to numerous effects in the cell [6].

Several studies highlight the antitumor activity of RA, particularly in the modulation of cell differentiation, metastasis, apoptosis, proliferation, migration, and invasion [7]. Due to its wide range of biological effects, RA is considered a promising anticancer drug. To date, the most successful use of RA in cancer therapy has been in the treatment of acute promyelocytic leukemia (APL) and precancerous lesions such as leukoplakia, actinic keratosis, and cervical dysplasia [8–10]. Unfortunately, the attempt to use RA to treat the most prevalent cancers, such as breast cancer (BC), has failed since it demonstrated controversial results [11, 12]. This fact emphasizes the need for studies focused on better understanding the mechanical and biological function of RA.

Currently, metastasis is the leading cause of death in BC patients, being the most challenging threat in treating advanced cancer [13, 14]. The prerequisite for cancer cells to metastasize is to acquire a highly migratory and invasive phenotype. Cell migration requires the continuous and orchestrated formation and turnover of focal adhesion sites (FAs) [15]. FAs are structures involved in the attachment of cells to extracellular matrix (ECM) and in the recruitment of cortactin, N-WASP, and Arp2/3 complex to the reorganization of actin fibers to form specialized structures that trigger cell motility [15, 16]. FAs are formed by several proteins, including focal adhesion kinase (FAK), Src, paxillin, talin, vinculin, and others [15]. FAK acts as a kinase and adapter molecule. It is considered a central regulator of cell migration since it is uniquely positioned at the convergence point of integrins and receptor tyrosine kinase signal transduction pathways, which transmit signals from the ECM to the cell cytoskeleton [17]. FAK is overexpressed in colorectal, lung, ovarian, prostate, and breast cancer and is associated with tumor metastatic behavior [18–20].

Due to the critical role of FAK in tumorigenesis, several pharmaceutical FAK inhibitors (FAKi) have been developed and are currently being tested in clinical trials (clinicaltrials.gov) [21]. It has been suggested that FAKi need to be combined with other agents as today they have not shown evidence of single-agent activity [22–24]. At present, there are no studies that focus on the combination of RA plus FAKi in BC therapy. We previously demonstrated that RA inhibits adhesion and migration in BC cells [25–27], cellular processes where FAK plays a key role. Based on this, we hypothesized that the combination of RA plus FAKi (RA+FAKi) could improve the treatment of advanced-stage BC patients. Thus, the goal of the present study was to determine the effect of RA administered in combination with FAKi on human BC cell adhesion and migration to increase the state of knowledge of signaling pathways triggered by RA, particularly in those fundamental processes necessary for the development of metastasis.

Materials And Methods

Cell culture and treatments

The human breast carcinoma cell lines SK-BR3, T-47D, and the human cervical carcinoma cell line HeLa were obtained from the American Type Culture Collection (ATCC). The murine mammary adenocarcinoma cell line LM3 was kindly provided by Instituto de Oncología Ángel H. Roffo [28]. SK-BR3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose, supplemented with 10% fetal

bovine serum (FBS), penicillin, and streptomycin. T-47D cells were grown in RPMI 1640 supplemented with L-glutamine, 10% FBS, penicillin, and streptomycin. LM3 cells were cultured in a minimum essential medium (MEM), supplemented with 10% FBS and gentamycin. HeLa cells were cultured in DMEM medium supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin. All cell lines were maintained at 37°C in an incubator with 5% CO₂. All-trans-retinoic acid (RA) was obtained from Sigma Chemical Co. RA stock solution was dissolved in dimethyl sulfoxide (DMSO) and maintained at 20°C, protected from light in an inert atmosphere. According to our previously published results, RA was used at a final concentration of 0.1 µM – 100 µM for 72 h [25, 26]. FAK inhibitor (FAKi, sc-203950) was from Santa Cruz Biotechnology and was diluted in cell culture medium to the final concentration of 1-2 µM. All experiments with retinoids were performed in reduced room light.

Bioinformatics

Basal gene expression study in eight cell lines

two microarray gene expression datasets (GSE70884 and GSE68651) were programmatically downloaded from the publicly available Gene Expression Omnibus database (ncbi.nlm.nih.gov/geo/) using the R *GEOquery* package [29]. The first dataset consists of seven BC cell lines (MCF-7, T-47D, ZR75.1, BT-474, SK-BR3, MDA-MB-468, MDA-MB-231) with three replicates each. The second dataset is composed of three replicates of the MCF-10A cell line. The Combat method from the SVA package was used to combine the datasets into a single one and perform batch effects corrections on the expression values [30, 31]. For expression profiling and differential gene expression (DGE) analysis, log₂ transformation of the expression values was applied. For boxplot construction, we used the same eight cell lines aforementioned and five genes involved in RA-antitumoral effects (RARA, RARB, RARG, SRC, PTK2). For the heatmap construction and cluster analysis, from the previously obtained expression matrix, five cell lines (MCF-10A, T-47D, SK-BR3, MDA-MB-468, MDA-MB-231) and 16 genes involved in different processes related to metastasis (CFL1, ACTR2, WASL, ACTR3, CDH1, VIM, MSN, TLN1, CTNNB1, VCL, DNM2, PXN, CTTN, MMP9, MMP2, and CDH2) were selected. A z-score of each gene was performed to visualize the differences in gene expression patterns between each cell line. The columns were sorted based on a hierarchical cluster with average linkage and Pearson's correlation distance. According to Silhouette dendrograms, samples were grouped into two major clusters and five minor ones representing each cell line. Differential gene expression analysis (DGE) was performed using the R *limma* package [32], comparing SK-BR3 vs. MCF-10A cell lines and T-47D vs. MCF-10A cell lines. In all cases, log₂ fold change values were obtained associated with exact p-values.

Gene-expression study in RA and control BC cell lines

a gene expression dataset (GSE103426) was programmatically downloaded from the publicly available Gene Expression Omnibus database (ncbi.nlm.nih.gov/geo/) using the R *GEOquery* package [29]. This dataset consists of two BC cell lines (MDA-MB-231, MDA-MB-468) with three replicates each, with or without RA treatment. Differential gene expression analysis (DGE) was performed using the R *limma*

package [32] comparing RA treated MDA-MB-231 vs. control MDA-MB-231 BC cell lines and RA treated MDA-MB-468 vs. control MDA-MB-468 BC cell line. In all cases, $\log_2$ fold change values were obtained associated with exact p-values.

Immunoblotting

Cell lysates were separated by SDS-PAGE and transferred into PVDF membranes. Antibodies used were: RAR α (sc-515796), RAR β (sc-552), RAR γ (sc-7387), Src (sc-5266), LDH (sc-133123), GAPDH (sc-47724) from Santa Cruz Biotechnology; and FAK (bd-610088) from BD Transduction Laboratories. Primary and secondary antibodies were incubated using the standard techniques. Immunodetection was accomplished using enhanced chemiluminescence and was recorded with a quantitative digital imaging system (Chemidoc XRS with Image Lab, Bio-Rad).

Cell adhesion assay

Cells were exposed to RA (1–10 μ M), FAKi (1 μ M) or their combination RA (1 μ M) plus FAKi (1 μ M) for 72 h. Fifteen thousand T-47D cells/well, fifty thousand LM3 cells/well, and twenty thousand HeLa cells/well were seeded into 96-well plates previously coated with 1% sterile gelatin (Sigma-Aldrich). Cells were incubated at 37°C for 2h. Non-adherent T-47D cells were removed by gentle washing with PBS. The attached cells were fixed with 4% paraformaldehyde and stained with 10% ethanol/crystal violet for 20 min. Images were captured and counted in ten randomly chosen fields per well using a Nikon Eclipse E200 microscope coupled to a high-resolution CCD digital camera, as previously described [25]. Cell adhesion was calculated as a percentage of attached treated cells compared to untreated cells.

Wound healing assay

A scratch wound assay was conducted to assess the influence of RA (1–10 μ M), FAKi (1 μ M) or their combination RA (1 μ M) + FAKi (1 μ M) for 72 h on cell migration. T-47D, LM3, and HeLa cells were seeded in 24-well plates and incubated until 80% confluence. Wounds were made in the monolayers by scratching the surface with a pipette tip (10 μ l) as uniformly and straight as possible. The cells were washed and the treatments were prepared. Ten μ M Cytosine β -D-arabinofuranoside hydrochloride (Sigma Aldrich), an inhibitor of DNA strand separation that prevents cell division, was added to each well. Cell migration was monitored for 72 h. Digital images from cells were taken with a 16X objective. The distance of migration was analyzed by phase-contrast microscopy and closed areas were quantified using ImageJ software. Cell migration was calculated as a percentage of migrated area in treated cells compared to untreated cells.

Cell viability

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-difeniltetrazol] (Sigma-Aldrich) was dissolved in DMSO to a final concentration of 5 mg/ml. The working solution was 0.5 mg/ml MTT. LM3 cells were seeded into 96-well plates at a density of forty-five thousand cells/well. Treatment with RA (0.01 – 100 μ M), FAKi (0.5 – 2 μ M), and/or the combination of both drugs was performed. After 72 h, the medium was removed and

the cells were incubated with 100 μ l MTT/well for 4 h. Then, MTT was removed and the formazan crystal rings were dissolved in 100 μ l DMSO. Absorbance at 570 nm was measured by using a microplate reader (MULTISKAN EX; Thermo Scientific). Cell viability was calculated as a percentage of viability in treated cells compared to untreated cells.

Cell immunofluorescence

LM3 cells were grown on coverslips at a density of twenty thousand cells/coverslip and exposed to RA (1 μ M), FAKi (1 μ M) or their combination RA (1 μ M) + (1 μ M) FAKi for 72 h. Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% triton for 5 minutes. Blocking was performed with 3% bovine serum albumin for 30 min at room temperature. Then, cells were incubated with an antibody against FAK (BD-610088) (BD Transduction Laboratories) overnight at 4°C. After washing, cells were incubated with goat anti-mouse IgG-Alexa Fluor 488 (A-11001, Invitrogen) for 90 min at room temperature. Then, cells were incubated with Texas Red-Phalloidin (Sigma-Aldrich) for 30 min. After washing, the nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and coverslips were mounted with Vectashield mounting medium (Vector Laboratories).

Immunofluorescence was visualized using a Nikon Eclipse E200 microscope and recorded with a high-resolution DP70 Olympus digital camera. Fluorescence quantification was performed by defining regions of interest using binary masks created from whole-cell staining with Texas-Red-Phalloidin with ImageJ software, as indicated in Kelley et al. (2018) [33].

Animals

The *in vivo* studies were conducted with female BALB/c mice, between 2 - 4 months old and 20 - 25 g weight, purchased from Instituto de Medicina y Biología Experimental de Cuyo (IMBECU, Mendoza, Argentina). They were randomly assigned into four groups and housed in boxes of five animals each in a climate-controlled room, with free access to food and water and a 12 h light/12 h darkness schedule. All animals have cared following the Guiding Principles in the Care and Use of Animal of the U.S. National Institute of Health. The Institutional Animal Care and Use Committee of the School of Medical Science, Universidad Nacional de Cuyo (UNCuyo), Mendoza, Argentina (Protocol approval N°63/2015) approved all procedures.

Groups were as follows:

- 1) CONTROL (indicated as CON): animals inoculated with LM3 cells received an empty subcutaneous silastic pellet.
- 2) RA: animals inoculated with LM3 cells received a subcutaneous silastic pellet containing RA (10 mg).
- 3) FAKi: animals inoculated with LM3 cells (pretreated with FAKi) and received an empty subcutaneous silastic pellet.
- 4) RA plus FAKi (indicated as RA+FAKi): animals inoculated with LM3 cells (pretreated with FAKi) and received a subcutaneous silastic pellet containing RA (10 mg).

Animals were euthanized by cervical dislocation at the experimental endpoint or when they showed signs of suffering (human endpoint): loss of > 20% of the initial weight, ulcers, or when one of the tumor diameters exceeds 10 mm. Survival data were recorded in each case. Cell viability was higher than 95% as determined by the trypan blue exclusion test whenever a cellular injection was performed. The order of injection of different groups was randomized to eliminate any difference that may bias the outcome.

Orthotopic tumor growth

Mice were injected subcutaneously into the fourth mammary gland with three hundred thousand LM3 cells, treated or not with FAKi (1 μ M for 72 h). Then, animals of groups 1 and 3 received a subcutaneous silastic pellet of slow-release containing RA (10 mg) and the animals of groups 2 and 4 received an empty subcutaneous silastic pellet. Mice were monitored daily and twice a week tumor diameters were measured with a sliding caliper. Tumor volume was calculated with the formula $D \times d^2/2$ (where d is the smallest diameter and D is the largest) for assessment of growth rate. Forty-five days postinoculation of tumor cells, mice were euthanized and necropsied to extract the tumors, which were fixed and embedded in paraffin for subsequent histopathological studies.

Experimental Lung Metastasis Assay

the animals of groups 1 and 3 received a subcutaneous silastic pellet containing RA (10 mg) and the animals of groups 2 and 4 received an empty subcutaneous silastic pellet. After a week, mice were inoculated in the tail vein with three hundred thousand LM3 cells, pretreated or not with FAKi (1 μ M for 72 h). Twenty-one days postinoculation of tumor cells, mice were euthanized and necropsied. The lungs were ablated and fixed in Bouin's solution to record the number of superficial lung nodules in each case under a dissecting microscope.

Histological Analysis

For immunohistochemistry, orthotopic tumors were fixed in 4% formalin, embedded in paraffin and 4 μ m sections mounted on glass slides. Antigen unmasking was carried out in 0.01 M citrate buffer (pH 6.0) at 100 ° C for 30 min. The blockade was carried out with 10% skim milk for 1 h. Primary antibody Ki-67 (15580, Abcam) was incubated overnight at 4°C. As a detection method, we used the biotin-streptavidin-peroxidase system. Diaminobenzidine was used as a chromogen substrate. Slides were lightly counterstained with hematoxylin and observed with an Eclipse E400 microscope (Nikon). We analyzed five randomly selected samples from each group. Five fields were randomly considered for scoring. A minimum of 500 cells per tissue was counted.

Statistical Analysis

Statistical analysis of the data was performed with one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple-Comparisons using GraphPad Prism 5.03 software. The Kaplan-Meier and log-rank tests were used to estimate survival time after treatments and to compare experimental groups. $P < 0.05$ was considered statistically significant. All values were expressed as mean $\pm$ standard error (SD) of

three independent experiments. All bioinformatics analyses were performed using R version 4.0.4 in a Windows environment with Intel Core i7 with 32 GB of RAM.

Results

Differential gene expression analysis of cancer-related genes between BC cells

Published results of different trials show that the use of retinoids in BC patients has controversial results. Since RA exerts its effects via RARs and that Src-FAK are critical proteins involved in cancer progression, we initially explored the expression profiles of RARA, RARB, RARG, SRC, and PTK2 in different types of human BC cells (T-47D, MCF7, ZR75.1, BT-474, SK-BR3, MDA-MB-231, and MDA-MB-468) and a normal breast cell line (MCF-10A). For this purpose, we performed a transcriptome analysis using two datasets from the publicly available Gene Expression Omnibus database (GSE70884 and GSE68651). We found that RARA (RAR α), SRC (Src), and PTK2 (FAK) are highly expressed in all breast cells. MDA-MB-231 and SK-BR3 have the lowest and highest level of RARA expression, respectively. RARB and RARG are expressed at similarly low levels in all breast cell lines (Fig. 1A).

We next compared the expression of sixteen genes between RA-resistant (MDA-MB-231 and MDA-MB-468), RA-sensitive (SK-BR3 and T-47D) BC cells, and a normal breast cell line (MCF-10A) using the previously obtained expression data. The selection of this set of genes was since they are biomarkers of different processes involved in BC metastasis such as MSN (moesin), CFL1 (cofilin) (*actin cytoskeleton remodeling/depolymerization process*); VCL (vinculin), TLN1 (talin), PXN (paxillin), DNMT2 (dynamin) (*FA dynamics*); CTTN (cortactin), WASL (family of the Wiskott Aldrich Syndrome Proteins, WASP), ACTR2 (Arp2 subunit), ACTR3 (Arp3 subunit) (*actin nucleation*); CDH1 (cadherin-1), VIM (vimentin), CDH2 (cadherin-2), CTNNB1 (β -catenin) (*Epithelial-Mesenchymal Transition, EMT*); and MMP2, MMP9 (matrix metalloproteinases 2 and 9) (*invasion/extracellular matrix disassembly*). All samples were sorted based on a hierarchical cluster algorithm with average linkage and Pearson's correlation distance. According to Silhouette dendrograms, analysis samples were grouped into two major clusters. In one branch, it groups MDA-MB-231 and MDA-MB-468 (RA-resistant) cell lines; in the other, it groups SK-BR3, T-47D (RA-sensitive), and MCF-10A (normal) cell lines. Figure 1B shows genetic similarities between RA-resistant BC cell lines and, on the other hand, between RA-sensitive BC cell lines and the normal ones. Furthermore, we observed that the expression of VIM, MSN, TLN1, and CTNNB1 is markedly different between RA-sensitive and RA-resistant BC cells. In RA-resistant cells, these genes are overexpressed; meanwhile, in RA-sensitive are underexpressed. We noted that the expression of these genes is similar between RA-sensitive cells and the normal breast cell line MCF-10A (Fig. 1B, *genes marked in blue*). MMP9 expression is low in RA-resistant compared with RA-sensitive and normal lines (Fig. 1B). Additionally, we found that the expression of CTTN and CDH2 is overexpressed in RA-resistant and normal breast cells compared to RA-sensitive (Fig. 1B, *genes marked in orange*). Altogether, this suggests that VIM, MSN, TLN1, CTNNB1, CTTN, MMP9, and CDH2 are promising candidates to be studied as markers of RA response.

To further elucidate whether genes are associated with RA-resistance, we performed a differential gene expression analysis in control and RA-treated MDA-MB-468 and MDA-MB-231 BC cells. In Figure 1C-D, both cells show the same behavior. RA administration (100 nM, 18 h) modulated the expression of all the genes analyzed, except ACTR2 and CFL1. Genes like WASL, CTTN, SRC, and DNM2 were upregulated (Fig. 1C-D, *genes marked in red*), while VIM, MSN, TLN1, PXN, VCL, ACTR3, and PTK2 were downregulated (Fig. 1C-D, *genes marked in green*). These results suggest that RA-resistance would reflect deregulation of most RA-target genes, mainly those encoding components from the signaling of the Src-FAK pathway.

Next, based on SK-BR3 and T-47D having a high genomic similarity concerning metastatic BC patients [34], we examined the differential gene expression between SK-BR3/T-47D and normal mammary cell line MCF-10A. We used a gene expression dataset from the publicly available Gene Expression Omnibus database (GSE103426). The volcano diagrams show that both BC cells exhibit a decreased expression of VIM, CDH2, and MSN compared to MCF-10A (Fig. 1E-F, *genes marked in green*). We also found that SK-BR3 has an increased expression of RARA, WASL, SRC, and MMP9 compared to MCF-10A (Fig. 1E, *genes marked in red*), while T-47D has an increased expression of CDH1 and PXN (Fig. 1F, *genes marked in red*).

RA reduces cell adhesion and migration in tumoral cells

To confirm the role of RA in inhibiting tumor progression, we first verified the expression of RARs, Src, and FAK in T-47D, SK-BR3, and LM3 by western blot analysis. We found that RAR α , RAR β , RAR γ , Src, and FAK are present in all BC cell lines analyzed (Fig. 2A).

Then, we evaluated the RA effect on cell adhesion and migration in human T-47D and murine LM3 BC cell lines. In both models, treatment with RA (1 - 10 μ M) for 72 h markedly decreased cell adhesion to an extracellular matrix-like surface by 58-79%, respectively, compared to the control group (Fig. 2B-E). Likewise, the administration of RA (1 - 10 μ M) during 72 h reduced cell migration by 52-88% in T-47D and by 25-35% in LM3 cells (Fig. 2H-K). Furthermore, we noted that RA inhibitory effects are extrapolated to other cancer cells. Treatment with RA (1 - 10 μ M) for 72 h in human cervical carcinoma cell line HeLa significantly inhibited cell adhesion (Fig. 2F-G) and migration (Fig. 2L-M) in a dose-dependent manner.

Treatment with RA plus FAKi reduces RAR α and FAK expression and decreases LM3 cells viability

Due to the fundamental role of RA and FAK in tumor progression, we next evaluated whether the combined treatment of RA with the specific inhibitor of FAK (FAKi) improves the inhibition of tumorigenesis. First, we tested the sensitivity of LM3 cells to drugs. We performed an MTT assay using a dose range of RA (0.01 – 100 μ M) and FAKi (0.5 - 2 μ M) and their combinations for 72 h. We observed that treatment with RA decreased cell viability in a dose-dependent manner. The IC₅₀ was reached with a dose of 100 μ M RA (Fig. 3A). The maximal dose tested of FAKi showed a significant decrease in cell viability of 38% compared with untreated cells. A synergistic effect was observed in the inhibition of cell viability when combined treatments were administered at the highest concentration (Fig. 3A). These results support the concept that LM3 BC cells are sensitive to RA and FAKi treatment.

Next, LM3 cells were treated for 72 h with RA (1 μ M), FAKi (1 - 2 μ M), and their combinations to analyze the expression of RARs and FAK (Fig. 3B). We found that all treatments induced downregulation of RAR α and FAK compared to the control. The RA+FAKi combinations resulted in higher downregulation than RA alone (Fig. 3B-C and F). On the other hand, we observed that the administration of RA induced upregulation of RAR β , while the treatment with FAKi (2 μ M) decreased its expression compared to the control group. Moreover, RA+FAKi reduced RAR β expression compared to RA but did not affect its expression compared to the control (Fig. 3B and D). Regarding RAR γ , we observed that RA and FAKi reduced its expression as a single agent compared to the control; however, their combinations did not affect it (Fig. 3B and E).

We also performed an immunofluorescence assay on LM3 cells treated with RA (1 μ M), FAKi (1 μ M), and their combination for 72 h to reveal the expression and subcellular localization of FAK. We visualized that FAK is homogeneously and diffusely localized throughout the cytoplasm in control cells, whereas treatment with RA and RA+FAKi triggers FAK nuclear relocalization (Fig. 3G). Furthermore, we observed in all treatments the longitudinal arrangement of actin fibers, typical characteristics of a static cell phenotype, suggesting that drug administration affects the reorganization of the actin cytoskeleton (Fig. 3G), preventing the cell from being able to drive its motor machinery impairing BC cell motility. In parallel, we also confirmed that the combined treatment significantly reduced FAK expression (mean intensity, pixel/area) (Fig. 3H).

RA and FAKi combination improve LM3 adhesion and migration inhibition

We tested the ability of LM3 cells to adhere to an ECM substrate after RA (1 μ M), FAKi (1 μ M), and their combination during 72 h. We showed that treatment with RA, FAKi, and RA + FAKi reduced cell adhesion compared to the control. The RA plus FAKi produced a more significant inhibition than RA alone (Fig. 4A-B). In parallel, we exposed cells to RA, FAKi, and RA+FAKi and we monitored cell motility for 72 h. We found a significant inhibition of cell migration in all conditions tested compared to the control, reaching the major inhibition of migration in the combined treatment (Fig. 4C-D).

RA plus FAKi reduces tumor growth and metastasis, increasing mice survival

Finally, we performed a murine *in vivo* model to determine correlation with the *in vitro* results. Tumor growth was evaluated by the orthotopic tumor growth assay consisting of inoculating murine LM3 cells, treated or not with FAKi (1 μ M, 72 h), in the mammary gland BALB/c mice bearing or not RA (10 mg)-subcutaneous pellet. We observed that RA and FAKi administered separately reduced the tumor growth, but the combined treatment induced a more potent inhibition (Fig. 5A-B). In concordance, RA and FAKi treatment increased mice survival, but only the combination RA+FAKi was statistically significant (Fig. 5C). In addition, we observed that in controls and in the FAKi group the tumors were histopathologically aggressive, even ulcerating the dermis. The treatment with RA and the combination RA+FAKi prevented the development of these highly proliferative tumors. Representative images of ulcers found in control animals (Fig. 5D-E and H) and tumors of combined treatments are shown (Fig. 5F-G). The total body

weight of mice was not different in the experimental groups compared to control, demonstrating a lack of RA-dependent toxicity (*data not shown*).

On the other hand, since Ki-67 is widely used in routine pathology as an established biomarker of cell division, to assess the proliferation rate of human BC tumors, we measured Ki-67 expression by immunohistochemistry in mice tumors. We found that the percentage of positive cells for Ki-67 was markedly lower in the treatments with FAKi and RA+FAKi compared to the control group (Fig. 5I-J).

Finally, we performed an experimental metastatic assay to analyze the effect of the RA+FAKi combination on the spread of tumor cells. LM3 cells, pretreated or not with FAKi (1 μ M, 72 h), were injected into the tail vein of mice bearing or not RA (10 mg)-silastic pellet. We found that RA significantly reduced metastatic lung dissemination. FAKi and the RA+FAKi combination presented a lower but non-significant number of lung nodules than the control group (Fig. 5K-L).

Discussion

Although RA has demonstrated a potent anticancer activity, its use in solid tumors is limited by its controversial behavior, emphasizing the need for a better understanding of the RA molecular mechanism. Thus, we evaluate whether a therapeutic approach based on RA administration combined with FAKi improves the inhibition of tumor progression since FAK is a pivotal kinase and scaffold protein that modulates cell adhesion and migration, processes involved in metastasis development.

Bioinformatics analysis confirmed that the expression of SRC and PTK2 is high in BC cells. The overexpression of Src and FAK in many cancers is well established and their correlation with a poor prognosis for patients [35, 19, 36, 37]. We also observed that RARA, encoding RAR α , the central receptor that mediates the antitumor activity of RA, was expressed at different levels within BC cells. This fact coincides with RA-sensitivity reported for BC cell lines, where SK-BR3 is considered RA-sensitive and MDA-MB-231 RA-resistant based on its high and low expression of RARA, respectively [38–40]. We also showed that RARB and RARG, encoding RAR β and RAR γ respectively, were uniformly and under-expressed in all BC. Similar results were obtained by Lu et al. (2005) [39]. This fact is not surprising since RAR β has been reported to act as tumor suppressors [41, 42]. RAR β is necessary to inhibit tumor proliferation and migration. It has been widely demonstrated the diminished of RARB mRNA due to epigenetic alterations in BC. It has been proposed as an epigenetic marker for BC diagnosis [25, 43, 44]. The role of RAR γ has not been extensively studied in the context of BC.

In addition, we showed that VIM, MSN, TLN1, CTNNB1, CTTN, MMP9, and CDH2 (encoding vimentin, moesin, talin, β -catenin, cortactin, metalloprotease-9, and N-cadherin, respectively) are differentially expressed between RA-resistant and RA-sensitive BC cells. The functional products of these genes are directly or indirectly modulated by the Src-FAK complex through interactions to trigger the metastatic process [24, 45].

In silico analysis in RA-resistant MDA-MB-231 and MDA-MB-468 BC cells showed that RA treatment regulated all genes tested despite being resistant to RA. Interestingly, RA downregulated VIM, MSN, and TLN1 genes previously demonstrated to be overexpressed in RA-resistant compared to RA-sensitive cells. In addition to these genes, RA reduced the expression of other genes involved in the adhesion/migration process, such as PXN, VCL, ACTR3, and PTK2. Despite lacking RARA receptors, and therefore being resistant to RA and insensitive to RA-induced decrease in proliferation, RA could inhibit the adhesion, migration, invasion, and, consequently, metastasis by reducing the expression of target genes involved in these events. Coyle et al. (2017) provided evidence that RA, in addition to the widely studied canonical mechanism, governs the expression of a vast number of genes in a RARE-independent way through the expression of additional RA-inducible transcription factors [46]. This result is promising for treating triple-negative BC that currently lacks a specific therapy; however, further studies on this issue are needed to substantiate this hypothesis.

Collectively, these data strongly support the idea that the combination of RA plus FAKi could improve the RA therapy in treating advanced-stage BC patients. Thus, we next examined the ability of RA+FAKi to inhibit cell adhesion and migration in BC cells. Numerous studies have found that RA reduces cell adhesion and migration in BC [26, 47, 25] and other types of cancer [48–50]. Our results confirm that RA inhibits cell adhesion and migration in T-47D, LM3, and HeLa cells. Disruption of cell adhesion and motility has also been reported after FAKi treatment in BC cells [51–54]. Our findings reveal that although RA and FAKi administered separately decrease adhesion and migration in LM3 BC cells, the novelty of our work is that their combination exerts a higher effect.

We also examined the ability of RA+FAKi on *in vitro* LM3 BC cell viability. We noted a synergistic effect when RA (100 μ M) and FAKi (2 μ M) were combined. Although no studies evaluated this combination, several reports demonstrated that RA and FAKi administered separately inhibit BC cells viability [55-58]. When we assessed the expression of RARs and FAK by western blot, we observed that RA induces the downregulation of RAR α and FAK. Coadministration with FAKi improves this downregulation. We have previously reported that RA treatment (72 h) promotes FAK downregulation [25]. Similarly, Dutta et al. (2009 and 2010) have demonstrated that RA treatment (20-30 μ M, 24-48 h) on MDA-MB-231 and MCF-7 cells triggers downregulation of FAK expression [59, 60]. They also reported that the administration of RA increases RAR expression, but they did not differentiate between RAR isoforms [60].

One of the most intriguing actions of these drugs is the control of distribution and expression of FAK, overexpressed in the majority of breast cancer subtypes and critical for the invasion and metastasis process. In this work, we observed that RA and FAKi reduce FAK expression, achieving a higher diminishment when cells were exposed to the combined RA+FAKi treatment. Interestingly, we also provide evidence that RA, FAKi, and the combined treatment inhibit the relocalization of FAK to the plasmatic membrane, leaving cells with a static phenotype. This phenomenon was observed when the treatment of RA and RA+FAKi promoted nuclear FAK translocation. Our group and other colleagues have reported that the individual use of RA and FAK inhibitor enables FAK shuttle between cytoplasm and the nucleus [61, 62, 26, 63]; however, the nuclear role of FAK is not completely clear. FAK in the cytoplasm controls

proliferation, adhesion, migration, and invasion in a kinase-dependent manner, while FAK in the nucleus acts in a kinase-independent way to regulate gene expression [63]. We suggest that when FAK is not found at FAs, it is impossible to trigger the migratory and invasive processes. In agreement with our previous findings, we propose an interesting regulatory mechanism in cell adhesion and migration, which are affected by the subcellular localization of FAK [26].

Another important finding of this work is the correlation between *in vitro* and *in vivo* results. In a murine BC model, we found that the administration of RA plus FAKi reduces tumors volume from the tenth day after orthotopic inoculation. Furthermore, we evidenced that mice administered with the combined treatment enhanced their survival compared to the control group. In concordance, other authors have demonstrated that RA inhibits the growth of LM3, MDA-MB-231, and MCF-7 orthotopic tumors [64–66]. Furthermore, we showed that the exposition of mice to the combined treatment decreases Ki-67 expression, indicating a more differentiated state and a reduction in tumor cellular proliferation. This result is consistent with previous works that demonstrated the molecular actions of RA and FAKi promoting tumor cell differentiation [67–69].

Finally, we evaluated the ability of RA and FAKi to inhibit tumor cells dissemination. RA administered as a single agent was effective in interrupting the spread of cancer cells to the lung. Previously, other groups have reported the ability of RA to inhibit metastasis [70–72]. In contrast, Berardi et al. (2014) and Bessone et al. (2020) did not observe differences between the control and RA-treated groups [64, 73]; however, they placed the RA patch five days before inoculation while we placed it one week previous to the injection.

In conclusion, our study reveals that RA-resistance would reflect the deregulation of most of the RA-target genes involved in cell adhesion, migration, and invasion. Despite the complexity of the retinoid signaling in BC, the approach based on RA administered in combination with FAKi may be an effective strategy for treating metastatic patients since we demonstrated that RA-sensibility could be exacerbated with FAKi coadministration. The combined treatment would inhibit malignant phenotype in BC cells by the disruption of cancer cell spread. Our findings also support the notion that the multifunctional protein FAK is a promising target for cancer therapy. Clinical trials are currently investigating treatment strategies using FAK inhibitors combined with chemotherapy, targeted therapy, or immunotherapy to increase FAKi efficacy [23]. Thus, it would be essential to select patients who could benefit from FAKi treatment alone or combination strategies.

Declarations

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author/s.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the School of Medical Science, Universidad Nacional de Cuyo, Mendoza, Argentina (Protocol approval N° 63/2015).

AUTHOR CONTRIBUTIONS

ACCG carried out different experiments, cell culture, and treatments.

JMM performed *in vivo* assays.

JMFM and FCMZ performed *in silico* analysis of RNA-Seq and microarray datasets.

FV performed immunoblottings assays.

IDU performed immunofluorescence assays.

ALR performed histology studies.

AMS was instrumental in funding the study and participated in the writing of the manuscript.

MIF planned and funded the project, supervised the experiments, wrote the manuscript.

All authors contributed to the article and approved the submitted version.

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References

1. Schenk T, Stengel S, Zelent A (2014) Unlocking the potential of retinoic acid in anticancer therapy. *Br J Cancer* 111 (11):2039-2045. doi:10.1038/bjc.2014.412

2. Dobrotkova V, Chlapek P, Mazanek P, Sterba J, Veselska R (2018) Traffic lights for retinoids in oncology: molecular markers of retinoid resistance and sensitivity and their use in the management of cancer differentiation therapy. *BMC Cancer* 18 (1):1059. doi:10.1186/s12885-018-4966-5
3. Lalevee S, Anno YN, Chatagnon A, Samarut E, Poch O, Laudet V, Benoit G, Lecompte O, Rochette-Egly C (2011) Genome-wide in silico identification of new conserved and functional retinoic acid receptor response elements (direct repeats separated by 5 bp). *J Biol Chem* 286 (38):33322-33334. doi:10.1074/jbc.M111.263681
4. Kumar S, Cunningham TJ, Duester G (2016) Nuclear receptor corepressors Ncor1 and Ncor2 (Smrt) are required for retinoic acid-dependent repression of Fgf8 during somitogenesis. *Dev Biol* 418 (1):204-215. doi:10.1016/j.ydbio.2016.08.005
5. Chambon P (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J* 10 (9):940-954
6. Al Tanoury Z, Piskunov A, Rochette-Egly C (2013) Vitamin A and retinoid signaling: genomic and nongenomic effects. *J Lipid Res* 54 (7):1761-1775. doi:10.1194/jlr.R030833
7. Giuli MV, Hanieh PN, Giuliani E, Rinaldi F, Marianecci C, Screpanti I, Checquolo S, Carafa M (2020) Current Trends in ATRA Delivery for Cancer Therapy. *Pharmaceutics* 12 (8). doi:10.3390/pharmaceutics12080707
8. Lo-Coco F, Avvisati G, Vignetti M, Thiede C, Orlando SM, Iacobelli S, Ferrara F, Fazi P, Cicconi L, Di Bona E, Specchia G, Sica S, Divona M, Levis A, Fiedler W, Cerqui E, Breccia M, Fioritoni G, Salih HR, Cazzola M, Melillo L, Carella AM, Brandts CH, Morra E, von Lilienfeld-Toal M, Hertenstein B, Wattad M, Lubbert M, Hanel M, Schmitz N, Link H, Kropp MG, Rambaldi A, La Nasa G, Luppi M, Ciceri F, Finizio O, Venditti A, Fabbiano F, Dohner K, Sauer M, Ganser A, Amadori S, Mandelli F, Dohner H, Ehninger G, Schlenk RF, Platzbecker U, Gruppo Italiano Malattie Ematologiche dA, German-Austrian Acute Myeloid Leukemia Study G, Study Alliance L (2013) Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med* 369 (2):111-121. doi:10.1056/NEJMoa1300874
9. Uray IP, Dmitrovsky E, Brown PH (2016) Retinoids and rexinoids in cancer prevention: from laboratory to clinic. *Semin Oncol* 43 (1):49-64. doi:10.1053/j.seminoncol.2015.09.002
10. Bushue N, Wan YJ (2010) Retinoid pathway and cancer therapeutics. *Adv Drug Deliv Rev* 62 (13):1285-1298. doi:10.1016/j.addr.2010.07.003
11. Garattini E, Bolis M, Garattini SK, Fratelli M, Centritto F, Paroni G, Gianni M, Zanetti A, Pagani A, Fisher JN, Zambelli A, Terao M (2014) Retinoids and breast cancer: from basic studies to the clinic and back again. *Cancer Treat Rev* 40 (6):739-749. doi:10.1016/j.ctrv.2014.01.001
12. Costantini L, Molinari R, Farinon B, Merendino N (2020) Retinoic Acids in the Treatment of Most Lethal Solid Cancers. *J Clin Med* 9 (2). doi:10.3390/jcm9020360
13. Gupta GP, Massague J (2006) Cancer metastasis: building a framework. *Cell* 127 (4):679-695. doi:10.1016/j.cell.2006.11.001
14. Singletary SE, Connolly JL (2006) Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual. *CA Cancer J Clin* 56 (1):37-47; quiz 50-31. doi:10.3322/canjclin.56.1.37

15. Nagano M, Hoshino D, Koshikawa N, Akizawa T, Seiki M (2012) Turnover of focal adhesions and cancer cell migration. *Int J Cell Biol* 2012:310616. doi:10.1155/2012/310616
16. Shortrede JE, Uzair ID, Neira FJ, Flamini MI, Sanchez AM (2016) Paxillin, a novel controller in the signaling of estrogen to FAK/N-WASP/Arp2/3 complex in breast cancer cells. *Mol Cell Endocrinol* 430:56-67. doi:10.1016/j.mce.2016.04.007
17. Zhao X, Guan JL (2011) Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev* 63 (8):610-615. doi:10.1016/j.addr.2010.11.001
18. Agochiya M, Brunton VG, Owens DW, Parkinson EK, Paraskeva C, Keith WN, Frame MC (1999) Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. *Oncogene* 18 (41):5646-5653. doi:10.1038/sj.onc.1202957
19. Cance WG, Harris JE, Iacocca MV, Roche E, Yang X, Chang J, Simkins S, Xu L (2000) Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes. *Clin Cancer Res* 6 (6):2417-2423
20. Miyazaki T, Kato H, Nakajima M, Sohda M, Fukai Y, Masuda N, Manda R, Fukuchi M, Tsukada K, Kuwano H (2003) FAK overexpression is correlated with tumour invasiveness and lymph node metastasis in oesophageal squamous cell carcinoma. *Br J Cancer* 89 (1):140-145. doi:10.1038/sj.bjc.6601050
21. Lv PC, Jiang AQ, Zhang WM, Zhu HL (2018) FAK inhibitors in Cancer, a patent review. *Expert Opin Ther Pat* 28 (2):139-145. doi:10.1080/13543776.2018.1414183
22. Schultze A, Fiedler W (2010) Therapeutic potential and limitations of new FAK inhibitors in the treatment of cancer. *Expert Opin Investig Drugs* 19 (6):777-788. doi:10.1517/13543784.2010.489548
23. Mohanty A, Pharaon RR, Nam A, Salgia S, Kulkarni P, Massarelli E (2020) FAK-targeted and combination therapies for the treatment of cancer: an overview of phase I and II clinical trials. *Expert Opin Investig Drugs* 29 (4):399-409. doi:10.1080/13543784.2020.1740680
24. Mousson A, Sick E, Carl P, Dujardin D, De Mey J, Ronde P (2018) Targeting Focal Adhesion Kinase Using Inhibitors of Protein-Protein Interactions. *Cancers (Basel)* 10 (9). doi:10.3390/cancers10090278
25. Flamini MI, Gauna GV, Sottile ML, Nadin BS, Sanchez AM, Vargas-Roig LM (2014) Retinoic acid reduces migration of human breast cancer cells: role of retinoic acid receptor beta. *J Cell Mol Med* 18 (6):1113-1123. doi:10.1111/jcmm.12256
26. Sanchez AM, Shortrede JE, Vargas-Roig LM, Flamini MI (2016) Retinoic acid induces nuclear FAK translocation and reduces breast cancer cell adhesion through Moesin, FAK, and Paxillin. *Mol Cell Endocrinol* 430:1-11. doi:10.1016/j.mce.2016.04.021
27. Vanderhoeven F, Redondo AL, Martinez AL, Vargas-Roig LM, Sanchez AM, Flamini MI (2018) Synergistic antitumor activity by combining trastuzumab with retinoic acid in HER2 positive human breast cancer cells. *Oncotarget* 9 (41):26527-26542. doi:10.18632/oncotarget.25480

28. Urtreger A, Ladeda V, Puricelli L, Rivelli A, Vidal M, Delustig E, Joffe E (1997) Modulation of fibronectin expression and proteolytic activity associated with the invasive and metastatic phenotype in two new murine mammary tumor cell lines. *Int J Oncol* 11 (3):489-496. doi:10.3892/ijo.11.3.489
29. Davis S, Meltzer PS (2007) GEOquery: a bridge between the Gene Expression Omnibus (GEO) and BioConductor. *Bioinformatics* 23 (14):1846-1847. doi:10.1093/bioinformatics/btm254
30. Johnson WE, Li C, Rabinovic A (2007) Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 8 (1):118-127. doi:10.1093/biostatistics/kxj037
31. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD (2012) The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 28 (6):882-883. doi:10.1093/bioinformatics/bts034
32. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43 (7):e47. doi:10.1093/nar/gkv007
33. Kelley JB, Paschal BM (2019) Fluorescence-based quantification of nucleocytoplasmic transport. *Methods* 157:106-114. doi:10.1016/j.ymeth.2018.11.002
34. Liu K, Newbury PA, Glicksberg BS, Zeng WZD, Paithankar S, Andrechek ER, Chen B (2019) Evaluating cell lines as models for metastatic breast cancer through integrative analysis of genomic data. *Nat Commun* 10 (1):2138. doi:10.1038/s41467-019-10148-6
35. Zou D, Yoon HS, Anjomshoaa A, Perez D, Fukuzawa R, Guilford P, Humar B (2009) Increased levels of active c-Src distinguish invasive from in situ lobular lesions. *Breast Cancer Res* 11 (4):R45. doi:10.1186/bcr2332
36. Elsberger B, Fullerton R, Zino S, Jordan F, Mitchell TJ, Brunton VG, Mallon EA, Shiels PG, Edwards J (2010) Breast cancer patients' clinical outcome measures are associated with Src kinase family member expression. *Br J Cancer* 103 (6):899-909. doi:10.1038/sj.bjc.6605829
37. Lark AL, Livasy CA, Dressler L, Moore DT, Millikan RC, Geradts J, Iacocca M, Cowan D, Little D, Craven RJ, Cance W (2005) High focal adhesion kinase expression in invasive breast carcinomas is associated with an aggressive phenotype. *Mod Pathol* 18 (10):1289-1294. doi:10.1038/modpathol.3800424
38. Centritto F, Paroni G, Bolis M, Garattini SK, Kurosaki M, Barzago MM, Zanetti A, Fisher JN, Scott MF, Pattini L, Lupi M, Ubezio P, Piccotti F, Zambelli A, Rizzo P, Gianni M, Fratelli M, Terao M, Garattini E (2015) Cellular and molecular determinants of all-trans retinoic acid sensitivity in breast cancer: Luminal phenotype and RARalpha expression. *EMBO Mol Med* 7 (7):950-972. doi:10.15252/emmm.201404670
39. Lu M, Mira-y-Lopez R, Nakajo S, Nakaya K, Jing Y (2005) Expression of estrogen receptor alpha, retinoic acid receptor alpha and cellular retinoic acid binding protein II genes is coordinately regulated in human breast cancer cells. *Oncogene* 24 (27):4362-4369. doi:10.1038/sj.onc.1208661
40. Schneider SM, Offterdinger M, Huber H, Grunt TW (2000) Activation of retinoic acid receptor alpha is sufficient for full induction of retinoid responses in SK-BR-3 and T47D human breast cancer cells.

41. Sun SY, Wan H, Yue P, Hong WK, Lotan R (2000) Evidence that retinoic acid receptor beta induction by retinoids is important for tumor cell growth inhibition. *J Biol Chem* 275 (22):17149-17153. doi:10.1074/jbc.M000527200
42. Xu Q, Jitkaew S, Choksi S, Kadigamuwa C, Qu J, Choe M, Jang J, Liu C, Liu ZG (2017) The cytoplasmic nuclear receptor RARgamma controls RIP1 initiated cell death when cIAP activity is inhibited. *Nat Commun* 8 (1):425. doi:10.1038/s41467-017-00496-6
43. Fang C, Jian ZY, Shen XF, Wei XM, Yu GZ, Zeng XT (2015) Promoter Methylation of the Retinoic Acid Receptor Beta2 (RARbeta2) Is Associated with Increased Risk of Breast Cancer: A PRISMA Compliant Meta-Analysis. *PLoS One* 10 (10):e0140329. doi:10.1371/journal.pone.0140329
44. Yari K, Rahimi Z (2019) Promoter Methylation Status of the Retinoic Acid Receptor-Beta 2 Gene in Breast Cancer Patients: A Case Control Study and Systematic Review. *Breast Care (Basel)* 14 (2):117-123. doi:10.1159/000489874
45. Zhao J, Guan JL (2009) Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev* 28 (1-2):35-49. doi:10.1007/s10555-008-9165-4
46. Coyle KM, Maxwell S, Thomas ML, Marcato P (2017) Profiling of the transcriptional response to all-trans retinoic acid in breast cancer cells reveals RARE-independent mechanisms of gene expression. *Sci Rep* 7 (1):16684. doi:10.1038/s41598-017-16687-6
47. Zanetti A, Affatato R, Centritto F, Fratelli M, Kurosaki M, Barzago MM, Bolis M, Terao M, Garattini E, Paroni G (2015) All-trans-retinoic Acid Modulates the Plasticity and Inhibits the Motility of Breast Cancer Cells: ROLE OF NOTCH1 AND TRANSFORMING GROWTH FACTOR (TGFbeta). *J Biol Chem* 290 (29):17690-17709. doi:10.1074/jbc.M115.638510
48. Cui J, Gong M, He Y, Li Q, He T, Bi Y (2016) All-trans retinoic acid inhibits proliferation, migration, invasion and induces differentiation of hepa1-6 cells through reversing EMT in vitro. *Int J Oncol* 48 (1):349-357. doi:10.3892/ijo.2015.3235
49. Woo YJ, Jang KL (2012) All-trans retinoic acid activates E-cadherin expression via promoter hypomethylation in the human colon carcinoma HCT116 cells. *Biochem Biophys Res Commun* 425 (4):944-949. doi:10.1016/j.bbrc.2012.08.038
50. Zuo L, Yang X, Lu M, Hu R, Zhu H, Zhang S, Zhou Q, Chen F, Gui S, Wang Y (2016) All-Trans Retinoic Acid Inhibits Human Colorectal Cancer Cells RKO Migration via Downregulating Myosin Light Chain Kinase Expression through MAPK Signaling Pathway. *Nutr Cancer* 68 (7):1225-1233. doi:10.1080/01635581.2016.1216138
51. Chauhan A, Khan T (2021) Focal adhesion kinase-An emerging viable target in cancer and development of focal adhesion kinase inhibitors. *Chem Biol Drug Des* 97 (3):774-794. doi:10.1111/cbdd.13808
52. Hao H, Naomoto Y, Bao X, Watanabe N, Sakurama K, Noma K, Motoki T, Tomono Y, Fukazawa T, Shirakawa Y, Yamatsuji T, Matsuoka J, Wang ZG, Takaoka M (2009) Focal adhesion kinase as potential target for cancer therapy (Review). *Oncol Rep* 22 (5):973-979. doi:10.3892/or_00000524

53. Lu Y, Sun H (2020) Progress in the Development of Small Molecular Inhibitors of Focal Adhesion Kinase (FAK). *J Med Chem* 63 (23):14382-14403. doi:10.1021/acs.jmedchem.0c01248
54. Parsons JT, Slack-Davis J, Tilghman R, Roberts WG (2008) Focal adhesion kinase: targeting adhesion signaling pathways for therapeutic intervention. *Clin Cancer Res* 14 (3):627-632. doi:10.1158/1078-0432.CCR-07-2220
55. Van heusden J, Wouters W, Ramaekers FC, Krekels MD, Dillen L, Borgers M, Smets G (1998) All-trans-retinoic acid metabolites significantly inhibit the proliferation of MCF-7 human breast cancer cells in vitro. *Br J Cancer* 77 (1):26-32. doi:10.1038/bjc.1998.5
56. Golubovskaya VM, Figel S, Ho BT, Johnson CP, Yemma M, Huang G, Zheng M, Nyberg C, Magis A, Ostrov DA, Gelman IH, Cance WG (2012) A small molecule focal adhesion kinase (FAK) inhibitor, targeting Y397 site: 1-(2-hydroxyethyl)-3, 5, 7-triaza-1-azoniatricyclo [3.3.1.1(3,7)]decane; bromide effectively inhibits FAK autophosphorylation activity and decreases cancer cell viability, clonogenicity and tumor growth in vivo. *Carcinogenesis* 33 (5):1004-1013. doi:10.1093/carcin/bgs120
57. Walsh C, Tanjoni I, Uryu S, Tomar A, Nam JO, Luo H, Phillips A, Patel N, Kwok C, McMahon G, Stupack DG, Schlaepfer DD (2010) Oral delivery of PND-1186 FAK inhibitor decreases tumor growth and spontaneous breast to lung metastasis in pre-clinical models. *Cancer Biol Ther* 9 (10):778-790. doi:10.4161/cbt.9.10.11433
58. Toma S, Isnardi L, Raffo P, Dastoli G, De Francisci E, Riccardi L, Palumbo R, Bollag W (1997) Effects of all-trans-retinoic acid and 13-cis-retinoic acid on breast-cancer cell lines: growth inhibition and apoptosis induction. *Int J Cancer* 70 (5):619-627. doi:10.1002/(sici)1097-0215(19970304)70:5<619::aid-ijc21>3.0.co;2-6
59. Dutta A, Sen T, Banerji A, Das S, Chatterjee A (2009) Studies on Multifunctional Effect of All-Trans Retinoic Acid (ATRA) on Matrix Metalloproteinase-2 (MMP-2) and Its Regulatory Molecules in Human Breast Cancer Cells (MCF-7). *J Oncol* 2009:627840. doi:10.1155/2009/627840
60. Dutta A, Sen T, Chatterjee A (2010) All-trans retinoic acid (ATRA) downregulates MMP-9 by modulating its regulatory molecules. *Cell Adh Migr* 4 (3):409-418. doi:10.4161/cam.4.3.11682
61. Lietha D, Cai X, Ceccarelli DF, Li Y, Schaller MD, Eck MJ (2007) Structural basis for the autoinhibition of focal adhesion kinase. *Cell* 129 (6):1177-1187. doi:10.1016/j.cell.2007.05.041
62. Lim ST, Chen XL, Lim Y, Hanson DA, Vo TT, Howerton K, Larocque N, Fisher SJ, Schlaepfer DD, Ilic D (2008) Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Mol Cell* 29 (1):9-22. doi:10.1016/j.molcel.2007.11.031
63. Zhou J, Yi Q, Tang L (2019) The roles of nuclear focal adhesion kinase (FAK) on Cancer: a focused review. *J Exp Clin Cancer Res* 38 (1):250. doi:10.1186/s13046-019-1265-1
64. Berardi DE, Bessone MI, Motter A, Bal de Kier Joffe ED, Urtreger AJ, Todaro LB (2015) Involvement of protein kinase C alpha and delta activities on the induction of the retinoic acid system in mammary cancer cells. *Mol Carcinog* 54 (10):1110-1121. doi:10.1002/mc.22181

65. Huang S, Chen Y, Liang ZM, Li NN, Liu Y, Zhu Y, Liao D, Zhou XZ, Lu KP, Yao Y, Luo ML (2019) Targeting Pin1 by All-Trans Retinoic Acid (ATRA) Overcomes Tamoxifen Resistance in Breast Cancer via Multifactorial Mechanisms. *Front Cell Dev Biol* 7:322. doi:10.3389/fcell.2019.00322
66. Wei S, Kozono S, Kats L, Nechama M, Li W, Guarnerio J, Luo M, You MH, Yao Y, Kondo A, Hu H, Bozkurt G, Moerke NJ, Cao S, Reschke M, Chen CH, Rego EM, Lo-Coco F, Cantley LC, Lee TH, Wu H, Zhang Y, Pandolfi PP, Zhou XZ, Lu KP (2015) Active Pin1 is a key target of all-trans retinoic acid in acute promyelocytic leukemia and breast cancer. *Nat Med* 21 (5):457-466. doi:10.1038/nm.3839
67. Gudas LJ, Wagner JA (2011) Retinoids regulate stem cell differentiation. *J Cell Physiol* 226 (2):322-330. doi:10.1002/jcp.22417
68. Kolev VN, Tam WF, Wright QG, McDermott SP, Vidal CM, Shapiro IM, Xu Q, Wicha MS, Pachter JA, Weaver DT (2017) Inhibition of FAK kinase activity preferentially targets cancer stem cells. *Oncotarget* 8 (31):51733-51747. doi:10.18632/oncotarget.18517
69. Luo M, Fan H, Nagy T, Wei H, Wang C, Liu S, Wicha MS, Guan JL (2009) Mammary epithelial-specific ablation of the focal adhesion kinase suppresses mammary tumorigenesis by affecting mammary cancer stem/progenitor cells. *Cancer Res* 69 (2):466-474. doi:10.1158/0008-5472.CAN-08-3078
70. Chansri N, Kawakami S, Yamashita F, Hashida M (2006) Inhibition of liver metastasis by all-trans retinoic acid incorporated into O/W emulsions in mice. *Int J Pharm* 321 (1-2):42-49. doi:10.1016/j.ijpharm.2006.05.008
71. Zhou Q, Xian M, Xiang S, Xiang D, Shao X, Wang J, Cao J, Yang X, Yang B, Ying M, He Q (2017) All-Trans Retinoic Acid Prevents Osteosarcoma Metastasis by Inhibiting M2 Polarization of Tumor-Associated Macrophages. *Cancer Immunol Res* 5 (7):547-559. doi:10.1158/2326-6066.CIR-16-0259
72. Grace VMB, Saranya S, Wilson DD (2021) Protective role of All Trans Retinoic Acid on B16F10 melanoma cell line metastasis in C57BL/6 mice by enhancing RAR- beta protein and homeostasis maintenance. *J Histotechnol* 44 (3):127-138. doi:10.1080/01478885.2021.1896291
73. Bessone MID, Berardi DE, Cirigliano SM, Delbart DI, Peters MG, Todaro LB, Urtreger AJ (2020) Protein Kinase C Alpha (PKCalpha) overexpression leads to a better response to retinoid acid therapy through Retinoic Acid Receptor Beta (RARbeta) activation in mammary cancer cells. *J Cancer Res Clin Oncol* 146 (12):3241-3253. doi:10.1007/s00432-020-03368-7

Figures

Figure 1

Figure 1

Expression of genes involved in metastasis process in a panel of BC cells. A) Boxplot showing the gene expression patterns of five genes of interest RARA, RARB, RARG, SRC, and PTK2 (encoding RAR α , RAR β , RAR γ , Src, and FAK respectively). Each color represents a breast cell line (MCF-10A, MCF-7, T-47D, ZR75.1, BT-474, SK-BR3, MDA-MB-468, MDA-MB-231). B) Gene expression heatmap of 16 genes involved in different processes related to metastasis. MCF-10A, T-47D, SK-BR3, MDA-MB-468, MDA-MB-231 cell lines

were used. Expression patterns of genes are represented in a green-red scale where low expression levels are in green and high expression levels in red. By a hierarchical clustering algorithm, samples were grouped into two major clusters and five minor ones that represent each cell line. C-D) Volcano plots of differential gene expression analysis achieved by limma R method. In the x-axis the log₂ fold change of RA-treated MDA-MB-231 vs. control MDA-MB-231 BC cell; and RA-treated MDA-MB-468 vs. control MDA-MB-468 BC cell. E-F) SK-BR3 vs. MCF-10A cells, and T-47D vs. MCF-10A cells. In the y-axis, the -log₁₀ of adjusted p value is shown. Genes with adjusted P-value < 0.05 are indicated above the horizontal dashed line. Upregulated genes are shown in red and down-regulated genes in green.

Figure 2

Figure 2

RA reduces cancer cell adhesion and migration. A) Whole-cell lysates from human BC cell lines T-47D, SK-BR3, and a murine BC cell line LM3 were evaluated to determine the expression of RARα, RARβ, RARγ, Src and FAK by western blot assay. GAPDH expression was used as a protein loading control. A representative blot is shown. B-C) T-47D, D-E) LM3, and F-G) HeLa cells were treated with RA (1 -10 μM) treatment for 72 h. After the treatment, cells were seeded into 96-well plates previously covered with

gelatin and a cell adhesion assay was performed. Representative images and percentages of adhered cells are shown. H-I) T-47D, J-K) LM3 and L-M) HeLa cells were exposed to RA (1-10 μ M) for 72 h and cell migration by wound-healing assay was performed. Representative images are shown. Gap closure was quantified by the use of the ImageJ software. Adhesion and migration values are presented as a percentage of control. * = $p < 0.05$ vs. control, CON. All experiments were performed in triplicates with consistent results.

Figure 3

Figure 3

LM3 cells viability and RARs-FAK expression. A) LM3 cells were treated with increasing doses of RA (0.01 - 100 μ M), FAKi (0,5 - 2 μ M), and their combinations (0.01 μ M RA + 0,5 μ M FAKi), (1 μ M RA + 1 μ M FAKi) and (100 μ M RA + 2 μ M FAKi) for 72 h. The results were expressed as a percentage (%) of surviving cells compared to the control (CON, 100 % survival). * = $p < 0.05$ vs. control, CON; # = $p < 0.05$ vs. RA B) LM3 cells were exposed with RA (1 μ M), FAKi (1 - 2 μ M) and their combinations (1 μ M RA + 1 μ M FAKi), (1 μ M RA + 2 μ M FAKi) for 72 h and expression of RAR α , RAR β , RAR γ , FAK were analyzed by western blot assay. LDH expression is shown as a loading control. C-F) Densitometric quantifications of RAR α , RAR β , RAR γ , and FAK bands. Intensity values were adjusted to the corresponding intensity values of LDH and then normalized to the control. * = $p < 0.05$ vs. control, CON; # = $p < 0.05$ vs. RA. All data shown were representative of three independent experiments. G) LM3 cells were exposed to RA (1 μ M), FAKi (1 μ M) or their combinations (1 μ M RA + 1 μ M FAKi) for 72 h. Immunofluorescence assay was performed, murine cells were stained with anti-FAK linked to Alexa Fluor 488 (green), filamentous actin was stained with phalloidin linked to Texas Red (red), and nuclei were counterstained with DAPI (blue). H) Quantification of mean FAK intensity in pixels per area in the different conditions. Results are expressed as a percentage of mean FAK intensity $\pm$ SD compared to the control cells. * = $p < 0.05$ vs. control, CON. # = $p < 0.05$ vs. RA. All experiments were performed in triplicate with consistent results; representative images are shown.

Figure 4

Figure 4

RA plus FAKi controls LM3 cell adhesion and migration. LM3 cells were treated with 1 μ M RA, 1 μ M FAKi or their combinations 1 μ M RA + 1 μ M FAKi for 72 h and cell adhesion and migration assay was performed. A-B) Representative images and percentages of LM3 cells adhered to gelatin and C-D) LM3 cell migration are shown. Gap closure was quantified by the use of the ImageJ software. Adhesion/migration results were expressed as a percentage of attached/migrated cells vs. control cells, CON. * = p 0.05 vs. control, CON. # = p 0.05 vs. RA. Measures are from three independent experiments.

Figure 5

Combination of RA plus FAKi in a murine experimental model. Orthotopic tumor growth assay was performed inoculating LM3 cells pretreated or not with FAKi (1 μ M, 72 h) into BALB/c mice. Animals also received a subcutaneous silastic pellet containing RA (10 mg) or an empty pellet. A) Tumor diameters were measured twice a week and used to calculate tumor volume. Each data point represents the mean $\pm$ SD, * = p 0.05 vs. control, CON. B) Representative images are shown. A dotted square indicates tumors. C) Kaplan-Meier analysis of animal endpoint survival. Each color represents a different experimental group: control group (black), RA group (red), FAKi group (green), and RA+FAKi group (blue). D-H) Effect of RA plus FAKi on histopathological features of LM3 orthotopic tumors. Tumors were fixed in 10% formalin and slides were stained with hematoxylin and eosin (T: Tumoral tissue, U: Ulcerated tissue, and A: Adipose tissue). D and F are images acquired at 100x magnification. E and G are the same sections with

a 400× magnification. H) Representative image of an ulcerated tumor. I-J) Images and the percentage of positive Ki-67-mammary tumor of LM3 cells compared to the control, CON; for each experimental condition are shown. * = $p < 0.05$ vs control, CON. K-L) Experimental metastasis assay was performed inoculating LM3 cells, pretreated or not with FAKi (1 μ M, 72 h), in the lateral tail vein of mice carrying a subcutaneous silastic pellet containing RA (10 mg) or an empty pellet. Twenty-one days post-inoculation, mice were sacrificed and the number of lung nodules was recorded. Each data point represents the number of lung nodules per animal. Each color denotes a different experimental group: control group (black), RA group (red), FAKi group (green), and RA+FAKi group (blue). Representative images are shown. The figure shows the results of one experiment representative of two independent assays. * = $p < 0.05$ vs control, CON.