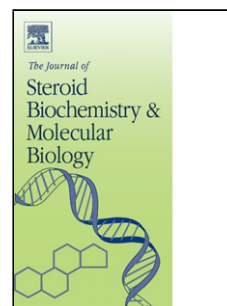# Journal Pre-proof

Vitamin D analogues exhibit antineoplastic activity in breast cancer patient-derived xenograft cells

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**Vitamin D analogues exhibit antineoplastic activity in breast cancer patient-derived xenograft cells**.

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**Graphical abstract**
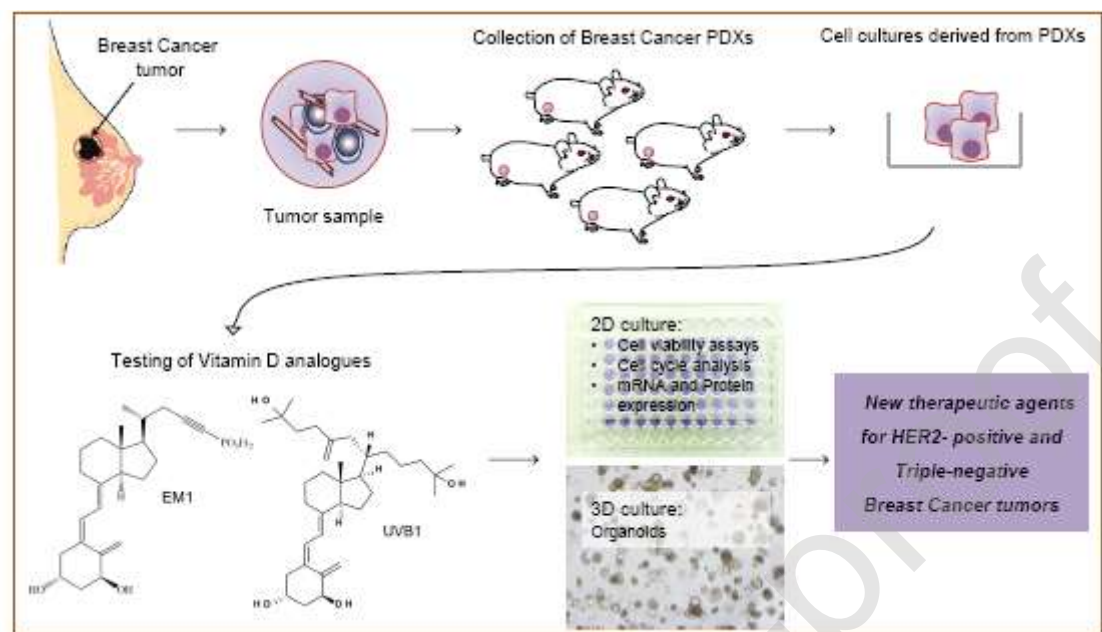


*Highlights*

- EM1 and UVB1 reduce the viability of cells derived from HER2-positive and TNBC PDXs
- UVB1 has antiproliferative actions in Trastuzumab-emtansine (T-DM1) resistant cells
- UVB1 impairs the organoids formation capacity in T-DM1 resistant cells
- UVB1 inhibits the growth of established organoids derived from T-DM1 resistant cells
- VDR expression in PDXs positively correlates with UVB1 antitumor response

**ABSTRACT**

Despite advances in breast cancer (BC) treatment, its mortality remains high due to intrinsic or acquired resistance to therapy. Several ongoing efforts are being made to develop novel drugs to treat this pathology with the aim to overcome resistance, prolong patient survival and improve their quality of life. We have previously shown that the non-hypercalcemic vitamin D analogues EM1 and UVB1 display antitumor effects in preclinical studies employing conventional cell lines and animal models developed from these cells. In this work, we explored the antitumor effects of EM1 and UVB1 employing BC cells derived from patient-derived xenografts (PDXs), which are a powerful preclinical tool for testing new drugs. We demonstrated that the analogues reduced the viability of HER2-positive and Triple Negative BC-PDXs. Moreover, using an in vitro model of acquired resistance to Trastuzumab-emtansine, UVB1 displayed anti-proliferative actions under 2D and 3D culture conditions. It inhibited both formation and growth of established organoids. In addition, a direct correlation between UVB1 antitumor effects and VDR expression in PDXs was found. In conclusion, all the results reinforce the potential use of these vitamin D analogues as antitumor agents to treat HER2-positive and Triple Negative BC.

**Keywords:** Breast Cancer; EM1; UVB1; Calcitriol; Analogue; Patient-Derived Xenograft; Organoids.

## 1. INTRODUCTION

Breast Cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer death in women worldwide [1]. BC is curable in ~70–80 % of patients with early stage (non- metastatic) and treatable in advanced (metastatic) stage. Treatment options for metastatic BC disease are addressed to prolong survival and control symptoms with low treatment- associated toxicity to maintain or improve quality of life.

Currently, treatment decisions are taken based on histological and molecular characteristics. Thus, tumors expressing estrogen receptor (ER) and/or

progesterone receptor (PR) are considered hormone receptor- positive BC and are treated with hormonal therapies. For those tumors that overexpress human epidermal growth factor receptor 2 (HER2), anti-HER2 therapies are available, including monoclonal antibodies (trastuzumab and pertuzumab), small molecule inhibitors (lapatinib, neratinib, and tucatinib) and antibody-drug conjugates (ADC) [2]. Trastuzumab emtansine (T-DM1) is an ADC composed of the humanized monoclonal antibody trastuzumab covalently linked to the microtubule-inhibitory agent DM1 [3]. T-DM1 has shown activity in patients with trastuzumab resistance [4,5]. Also, FDA has recently approved T-DM1 as adjuvant therapy in patients with HER2-positive early breast cancer who have residual invasive disease after neoadjuvant taxane- and trastuzumab-based treatment [6,7]. However, these strategies of treatment are ineffective for triple-negative BC (TNBC), which does not express ER or PR and lacks HER2 overexpression or amplification. Although TNBC patients respond to chemotherapeutic agents such as taxanes and anthracyclines better than other subtypes of BC, prognosis remains poor [8]. In addition, the development of resistance to BC therapies that are initially effective is commonly observed [9, 10]. Therefore, the identification of novel compounds to treat BC is needed.

Vitamin D Receptor (VDR), a nuclear transcription-regulating factor that belongs to the steroid hormone superfamily, is involved in cell growth and differentiation in normal and BC tissue. Calcitriol is the most active form of Vitamin D that binds to VDR. Despite antitumor properties of calcitriol reported with this compound, its clinical use is partly limited due to the risk of developing adverse effects, such as hypercalcemia, at the effective dose required for antitumor activity [11]. Therefore, VDR agonists with low calcemic activity which retain or even increase the calcitriol-anticancer properties are being developed. Our group has synthetized two novel non-hypercalcemic vitamin D analogues, UVB1 and EM1, that have demonstrated promising antitumor effects in preclinical studies employing cell lines cultured under 2-dimensional (2D) conditions and in vivo models generated from these cells [12,13,14,15,16].

In the current work we focused on the study of the antineoplastic effects of UVB1 and EM1 on cells derived from patient-derived xenografts (PDXs) cultured under 2D and 3D conditions. These models recapitulate the

heterogeneity of BC tumors [17], being a powerful preclinical tool for testing new drugs. We demonstrated that the analogues reduced the viability of HER2-positive and TN BC-PDXs. Interestingly, using an in vitro model of acquired resistance to T-DM1, we showed that UVB1 displays anti-proliferative effects in 2D and 3D culture conditions. These results reinforce the potential use of these vitamin D analogues as antitumor agents to treat HER2-positive and TNBC.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Reagents

EM1, UVB1 and calcitriol (Sigma-Aldrich, D1530) were reconstituted as previously described [12,13]. Briefly, they were dissolved in 100% HPLC-grade isopropanol (vehicle) and stored protected from light at –20°C. Compounds were diluted in the culture medium to reach the required concentrations to perform the assays. The maximal concentration of vehicle used in this study had no effect on cell viability.

### 2.2. Cell culture

All cells, except HCC1954 cell line, were cultured in Dulbecco's Modified Eagle's Media (DMEM)/F12 (Sigma) supplemented with 10 % (v/v) fetal bovine serum (FBS, Gibco), 1 % (v/v) L-glutamine (Gibco), 1 % (v/v) Penicillin / Streptomycin (Gibco,) at 37ºC with 5 % $CO_2$ in humidified air atmosphere. HCC1954 cells were maintained in Roswell Park Memorial Institute (RPMI-1640, Sigma) medium supplemented with 10 % FBS, L-glutamine, penicillin and streptomycin. HCC1954 (#CRL-2338) and SK-BR-3 (#HTB-30) were from the American Type Culture Collection.

All cell cultures derived from PDXs were generated in Vall d'Hebron Institute of Oncology (VHIO). In vivo models were established with the approvals of the Vall d'Hebron University Hospital ethic committee and of the Ethical Committee for the Use of Experimental Animals at the VHIO. To this end, fragments of human BC tissues were implanted into the fourth fat pad of NOD.CB17-Prkdcscid/J (NOD/SCID) mice. Depending on tumor size, one to two mice were employed per sample to implant the patient biopsies. When tumors reached 1000 mm$^3$, mice were sacrificed. Tumors were excised and cut into the smallest pieces possible, digested in collagenase plus hyaluronidase for 1 h at 37 C with

shaking at 80 rpm, washed and resuspended in DMEM/F12 supplemented with 10 % FBS, L-glutamine and Penicillin/Streptomycin.

PDX118 and PDX554 were generated from skin and brain metastatic BC tumor samples of a HER2-positive BC tumor, respectively. PDX410, PDX570, PDX575, PDX347, PDX454 and PDX549 were originated from a biopsy of the primary TNBC tumor.

R200 is an in vitro model of acquired resistance to T-DM1. It was generated as previously described [18]. Briefly, PDX118 cells were continuously exposed to increasing concentrations of T-DM1 over a period of 3 months. The started concentration used was 20 ng/mL and it was progressively doubled every 10 days until 200 ng/mL was achieved. This model was tested for resistance in vitro following a dose response study of T-DM1 for a recent review of the complete characterization of this in vitro resistant model, see [18].

### 2.3. Cell viability assays

### 2.3.1. Crystal violet staining assays

Cells were seeded into 96-well plates in 100 µl of complete medium and treated as indicated in the corresponding figures. After the time of treatment, medium was removed and adherent cells were fixed in 10 % paraformaldehyde for 10 min, then washed thrice in distilled water and stained with 0.1 % crystal violet for 30 min. After washing, the stain was dissolved with 10% acetic acid and subsequently quantified with VICTOR3 Multilabel Plate Reader at 560 nm. Data obtained with crystal violet staining (optical density per well) were expressed as a percentage of control. The experiments were repeated at least twice and performed in triplicate.

### 2.3.2. Automatic cell counting assays

The same number of viable PDX118 cells ($3 \times 10^5$ cells) treated with UVB1 or vehicle ($10^{-7}$ M, 120 h) were seeded into 60 mm dishes and allowed to grow in complete medium without any stimuli. After 4 days, cell viability was measured by Vi-CELL XR automatic analyzer (Beckman Coulter). The experiments were performed in triplicate.

### 2.4. Cell cycle analysis

Human PDX118 parental and resistant cells treated with UVB1 ($10^{-7}$ M) or vehicle during 96 h were collected, washed twice with ice-cold PBS, fixed with ice-cold 70% ethanol and preserved at -20°C at least 24 h. Then, cells were washed with PBS, stained with 50 µg/mL Propidium Iodide (PI, Roche) plus RNase A (100 µg/mL) and analyzed for DNA content by FACScan flow cytometry (Becton Dickinson) with Cell Quest software (Becton Dickinson, Heidelberg, Germany). At least 100,000 cells were analyzed for each sample and the experiment was performed in triplicate.

### 2.5. Western blot analysis

PDX118 parental cells treated with UVB1 or vehicle ($10^{-7}$ M, 96 h) and cells derived from HER2-positive and TNBC PDXs cultured to 70–80% confluence were lysed in lysis buffer containing 2 % TRIS 1 M, 1 % Triton-X 100, 0.5 M EDTA 0.5 M, 2 % sodium chloride 1 M and protease inhibitors for 30 min on ice and centrifuged at maximal speed for 10 min at 4°C. Protein extracts were quantified by Bradford assay using a Jasco V-630 spectrophotometer. The same amount of protein was resolved by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore). Membranes were subsequently blocked with 2 % non-fat milk in PBS for 30 min at room temperature and further incubated at 4°C overnight with primary antibodies. Then, membranes were washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 90 min at room temperature. Protein bands were visualized using the enhanced chemiluminescence (ECL) method. Representative blots from at least three independent experiments are shown in the figures.

Primary antibodies recognizing human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#Ab128915, Abcam), Cyclin D1 (#92G2, Cell Signaling Technology), p21 (sc-397, Santa Cruz Biotechnology), p27 (#610241, BD Biosciences) and VDR (C-20, sc-1008, Santa Cruz Biotechnology) were used. As secondary antibodies, we used ECL rabbit IgG, HRP–linked whole antibody (from donkey, #NA934–1ML, Amersham GE Healthcare) and ECL mouse IgG, HRP-linked whole antibody (from sheep, #NA931, Amersham GE Healthcare).

### *2.6. CYP24A1 and VDR mRNA expression*

Cells derived from PDX118, R200 and PDX570 were treated with vehicle, UVB1, EM1 or calcitriol in triplicate ($10^{-7}$ M, 96 h). Total RNA isolation from treated cells was performed as recommended by the supplier (RNeasy Mini Kit, Qiagen, Hilden, Germany) and mRNA was reverse-transcribed to cDNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with Taqman primers (Applied Biosystems) for CYP24A1 (Hs00167999_m1), VDR (Hs01045843_m1) and GAPDH (Hs02758991_g1) using an ABI 7900HT Sequence Detection System. Each sample was assayed in triplicate. The CT (Cycle Threshold) values of the real-time PCR were extracted from each assay with SDSv2.0 software tool (Applied Biosystems) and analyzed using DataAssist Software (Applied Biosystems) following the manufacturer's instructions. CT values of target gene were normalized with the CT value of the housekeeping gene GAPDH and data were expressed relative to vehicle-treated cells.

### *2.7. Organoid culture*

Organoids were generated using R200 cells in culture with complete medium DMEM/F12.

On one hand, to evaluate the inhibition capacity of organoid formation by UVB1, single cells ($5 \times 10^3$ or $5 \times 10^4$ cells/ well) were seeded into 48-well plates in drops of Matrigel (100 %, #354234, BD) and UVB1 or vehicle treatment ($10^{-7}$ M) were added to culture medium. Medium was removed every two days and replaced by drugs treatment. After 7 or 14 days, culture medium was removed and wells containing organoids embedded in Matrigel were washed with PBS and trypsinized to disaggregate to single cells. Then, cell viability of each condition was measured by CellTiter-Glo® Luminescent Kit (Promega). This is a method to determine the number of viable cells based on quantitation of the ATP present, which is directly proportional to the number of cells present in the well. The experiments were repeated twice and performed in triplicate. Results were checked by automatic cell counting.

On the other hand, to evaluate UVB1 inhibition of established organoid growth, $1 \times 10^3$ R200 cells were seeded into 48-well plates in 100 % of Matrigel and allowed to grow until organoids formation (11 days). Then, they were treated with vehicle or UVB1 ($10^{-7}$ and $10^{-6}$ M), changing the treatments every two days. After 14 days, organoids from each condition were trypsinized and counted by Fluorescence-activated cell sorting (FACS).

### 2.8. Statistical analyses

GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Cell viability and cell cycle assays were analyzed with two-way Analysis of Variance (ANOVA) and Bonferroni post-tests. The assays performed to evaluate the inhibition of the capacity of organoid formation and the growth of established organoids were analyzed by unpaired t-test and one-way ANOVA and Bonferroni post-test, respectively. The correlation coefficient was calculated using Pearson's test based on the normal distribution of the variables. mRNA and protein expression were analyzed by one-way ANOVA and Bonferroni post-test. To establish whether or not each parameter group represented a Gaussian distribution, a D'Agostino–Pearson Omnibus normality test was applied. Results were considered to be statistically significant at a $p < 0.05$.

## 3. RESULTS

### Vitamin D analogues UVB1 and EM1 reduce cell viability of human HER2-positive BC cell lines.

We had previously demonstrated that the analogues UVB1 and EM1 decreased cell viability of cell lines coming from different tumor types (colon cancer, head and neck squamous cell carcinoma, glioblastoma multiforme) [12,13,15,16]. Additionally, EM1 displayed anti-metastatic effect [14] in an animal model developed from hormone-independent HER2-positive LM3 cell line, which is derived from a murine mammary adenocarcinoma [19]. Taking into account these previous results, we proposed to investigate the antitumor activity of these analogues in the viability of human HER2-positive BC cell lines and then on cells derived from PDXs cultured under 2D and 3D conditions. To this end, we initially evaluated the effects of the analogues on two human

HER2-positive BC cell lines, HCC1954 and SK-BR-3. As shown in Figure 1, UVB1 reduced the cell viability of both BC cells lines, while EM1 decreased the viability of SK-BR-3 cells only at the concentration of $10^{-6}$ M.
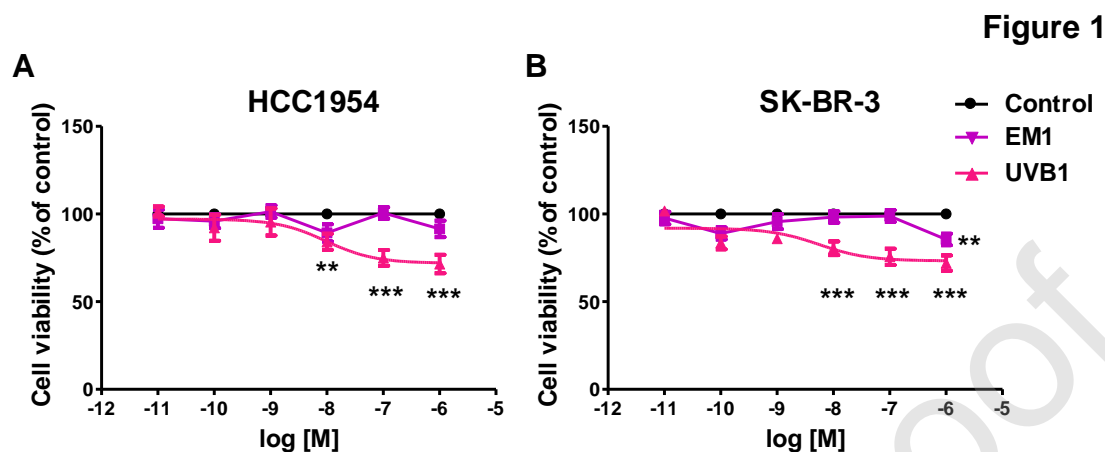
**Figure 1**



**Figure 1. Vitamin D analogues reduce cell viability of HER2-positive BC cell lines.** Concentration-response curves obtained from cells exposed to vehicle, UVB1 or EM1 for 120 h and stained with crystal violet. The results were expressed as percentage of vehicle-treated cells. Data points represent mean ± SEM from three independent experiments. Two-way ANOVA and Bonferroni post-test was applied; **$p<0.01$ and ***$p<0.001$.

## Vitamin D analogues UVB1 and EM1 decrease the viability of cells derived from HER2-positive PDXs.

In order to test the antitumor effects of the analogues on PDXs cell viability, and compare the effects with those obtained in cell lines, we performed viability assays using two different HER2-positive PDXs: PDX118 and PDX554 generated from skin and brain metastatic BC tumor samples, respectively. As shown in Figure 2, the UVB1 analogue decreased the viability of both PDXs and EM1 only reduced the viability of PDX554 at $10^{-6}$ M (Figure 2 A and B).

Also, we tested the effects of both analogues on R200 cells. These cells are an in vitro model of acquired resistance to T-DM1, the second-line therapy for HER2-positive BC [20]. It was generated from PDX118 that was chronically treated with increasing concentrations of T-DM1. Interestingly, we obtained similar findings compared to parental cells. The analogue EM1 did not affect the viability of R200 cells whereas UVB1 significantly reduced it (Figure 2 C and D).
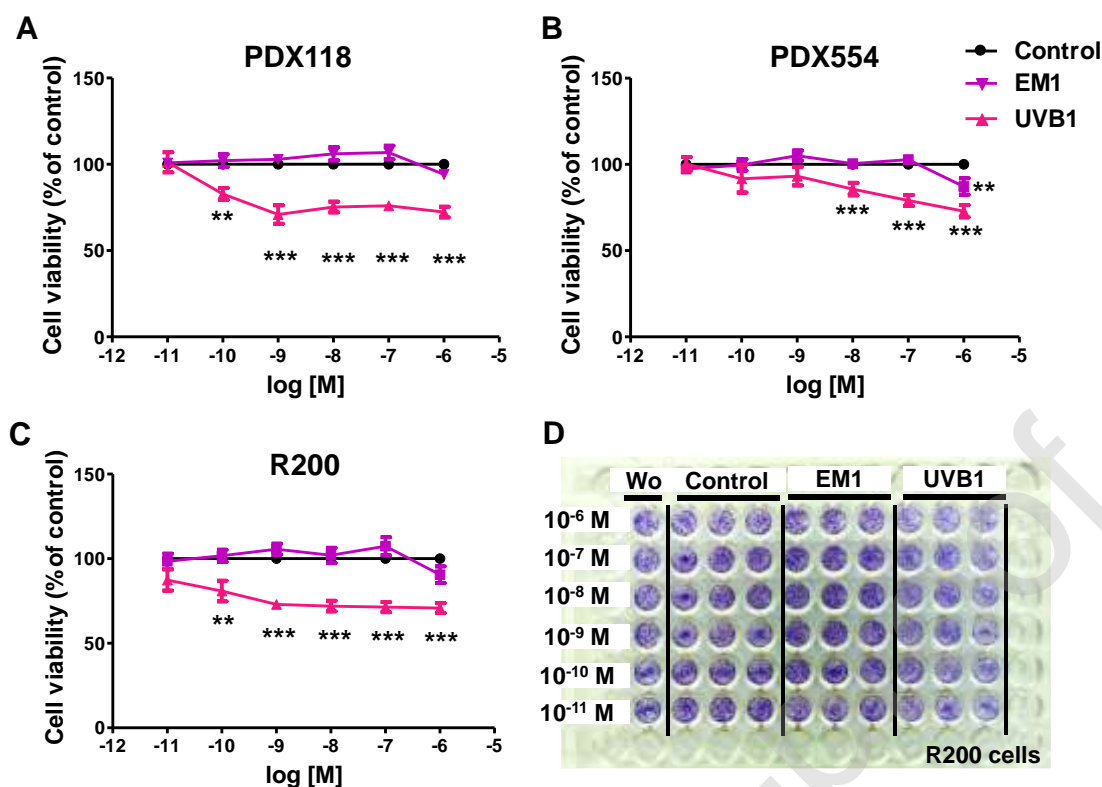
Figure 2



**Figure 2. Vitamin D analogues exhibit antitumor effect on cells derived from HER2-positive PDXs**. Concentration-response curves obtained from A) PDX118, B) PDX554 and C) R200 cells exposed to vehicle, UVB1 or EM1 for 120 h and stained with crystal violet. D) Photograph of a 96-well plate stained with crystal violet at the end point. Wo: without treatment. The results were expressed as percentage of vehicle-treated cells. Data points represent mean ± SEM from three independent experiments. Two-way ANOVA and Bonferroni post-test was applied; **p<0.01 and ***p<0.001.

All cell viability results show that UVB1 has a potent antineoplastic effect on HER2-positive BC cells and PDXs, and a stronger effect than EM1. Therefore, we investigated the cellular mechanisms underlying the antitumor effect of UVB1 using PDX118 and R200 cells. To this end, we performed cell cycle analysis of propidium iodide-stained cells treated with UVB1 or vehicle. As depicted in Figure 3 A and B, an arrest of PDX118 cells in G0/G1 phase was observed after treatment with UVB1 (85.99 ± 1.10 % of UVB1-treated cells vs 70.45 ± 3.57 % of vehicle-treated cells; ***p<0.001). Similar results were obtained with R200 cells, in which UVB1 analogue inhibited cell proliferation by an induction of cell cycle arrest in G0/G1 phase (83.04 ± 1.91 % of UVB1-

treated cells vs 75.74 ± 0.85 % of vehicle-treated cells; ***p<0.001). In concordance with cell cytometry results, a reduction of the cell cycle regulator Cyclin D1 expression was detected by Western Blot (Figure 3C), although no significant differences were observed in both p21 and p27 expression. As expected, EM1-treated cells showed the same levels of cyclin D1, p21 and p27 than control cells (Supplementary Figure 1).
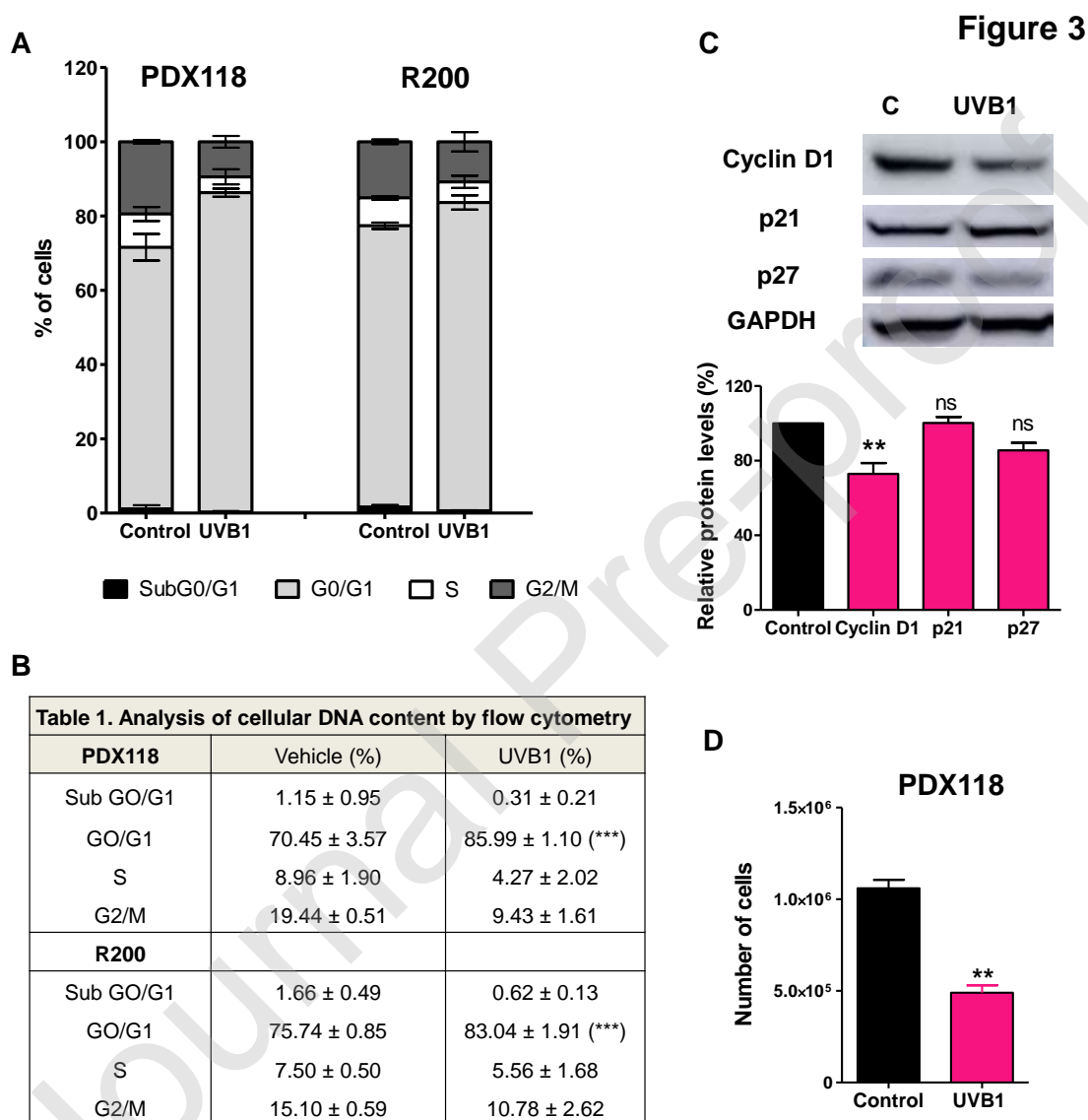


**Figure 3. UVB1 induces cell cycle arrest in HER2-positive BC cells derived from PDXs.** A) Cell cycle analysis of PDX118 cells treated with UVB1 ($10^{-7}$ M, 96 h) or vehicle. Cells were stained with PI and analyzed for DNA content with FACScan Calibur Becton Dickinson. Data points represent means ± SD from three replicates. ANOVA and Bonferroni post-test was applied, ***p<0.001 respect to vehicle. B) The table shows the percentage of PDX118 parental and R200 cells in each phase of cell cycle analyzed by flow cytometry. C) Western blot analyses to detect cyclin D1, p21 and p27 expression in PDX118 cells treated with UVB1 ($10^{-7}$

M) or vehicle for 96 h. The graph shows the densitometry of bands of at least three independent experiments. D) PDX118 cells were treated with vehicle or UVB1 ($10^{-7}$ M, 120 h), seeded in culture dishes and allowed to grow to evaluate if the effect of the analogue on proliferation is maintained over time. The results were expressed as number of cells at the end of the experiment. The graph shows mean ± SD from three replicates. Unpaired t test was applied; **p<0.01.

## Supplementary Figure 1

| | C | UVB1 | EM1 |
|---|---|---|---|
| Cyclin D1 | | | |
| p21 | | | |
| p27 | | | |
| GAPDH | | | |
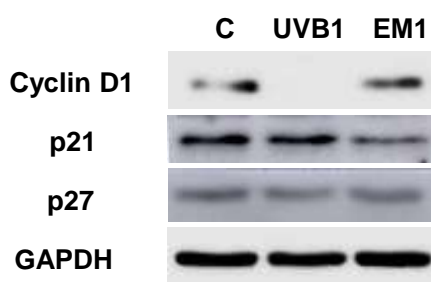
**Supplementary Figure 1.** Western blot analyses to detect cyclin D1, p21 and p27 expression in PDX118 cells treated with UVB1, EM1 ($10^{-7}$ M) or vehicle for 96 h.

In order to test if the anti-proliferative effect of UVB1 on PDX118 cells was maintained in absence of the analogue, we seeded 3 x $10^5$ cells pre-treated with UVB1 or vehicle in culture plates and allowed to growth in the absence of the compounds. After four days, cells were counted. We observed that control-treated cells continued dividing (1,060,000 ± 113.14 cells) while analogue-treated cells remained arrested (490,000 ± 98.99 cells) (Figure 3D). In fact, control-treated cells doubled 3.5 times while UVB1-treated cells did it 1.6 times in 4 days. This represents a 53.8 % of cell inhibition, suggesting that UVB1 anti-proliferative effect maintains over time, at least, during four days after treatment.

## UVB1 impairs organoids formation capacity in T-DM1 resistant cells.

When cells are placed within a hydrogel, often Matrigel, and in presence of suitable exogenous factors, the system mimics the tumor microenvironment present in vivo allowing cell-extracellular matrix interactions [21]. These characteristics make this model an attractive system to evaluate the antitumor potential of a novel compound in 3D culture conditions. Therefore, given our

previous results that UVB1 exerted antitumor effect in TDM-1 resistant cells, we embedded R200 cells in Matrigel, in presence of UVB1 in the culture medium and allowed cells to grow for 7 (Figure 4A) or 14 (Figure 4B) days. Confirming the previous results obtained under 2D conditions, we observed a remarkable inhibition in the capacity of R200 cells to establish organoids at both 7 and 14 days after UVB1 treatment. The percentage of inhibition was 57.58 ± 4.24 % at day 7 and 95.56 ± 0.51 % at day 14 after analogue treatment. Next, we tested if UVB1 could impair the growth of established organoids in order to mimic already established tumors in vivo.
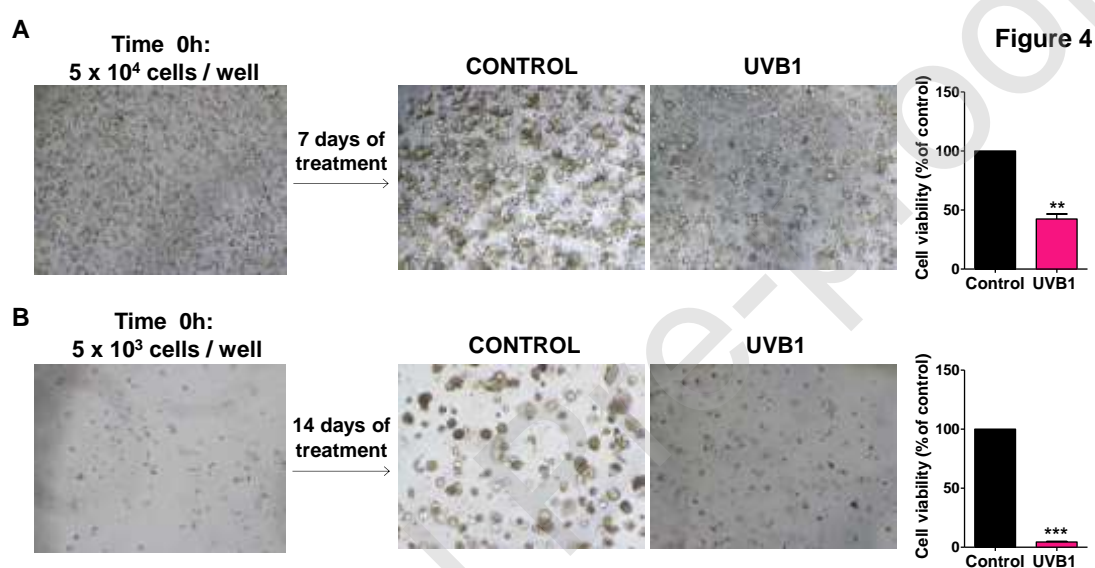


**Figure 4. UVB1 reduces tumor formation capacity of R200 cells grown under 3D culture conditions**. R200 single cells were seeded in Matrigel and treated with UVB1 ($10^{-7}$ M) during A) 7 days and B) 14 days. The kit CellTiter-Glo® was used to measure cell viability at the end of the treatment. Representative micrographs of the conditions are shown (x40). The assay was performed in triplicate. Unpaired t test was applied. **$p<0.01$; ***$p<0.001$.

## UVB1 inhibits the growth of established organoids generated from T-DM1 resistant cells.

We tested the effect of the analogue on established organoids obtained after 10 days of seeding R200 cells in Matrigel. These structures were treated with vehicle or UVB1 at two concentrations ($10^{-7}$ and $10^{-6}$ M). After 14 days of treatment, we detected a significant reduction in organoids growth at both

concentrations tested, compared to control (UVB1 = 64.36 ± 15.75 % and 0.02 ± 0.03 % at $10^{-7}$ and $10^{-6}$ M, respectively; Figure 5).

**Figure 5**

| CONTROL | UVB1 $10^{-7}$ M | UVB1 $10^{-6}$ M |
|---|---|---|



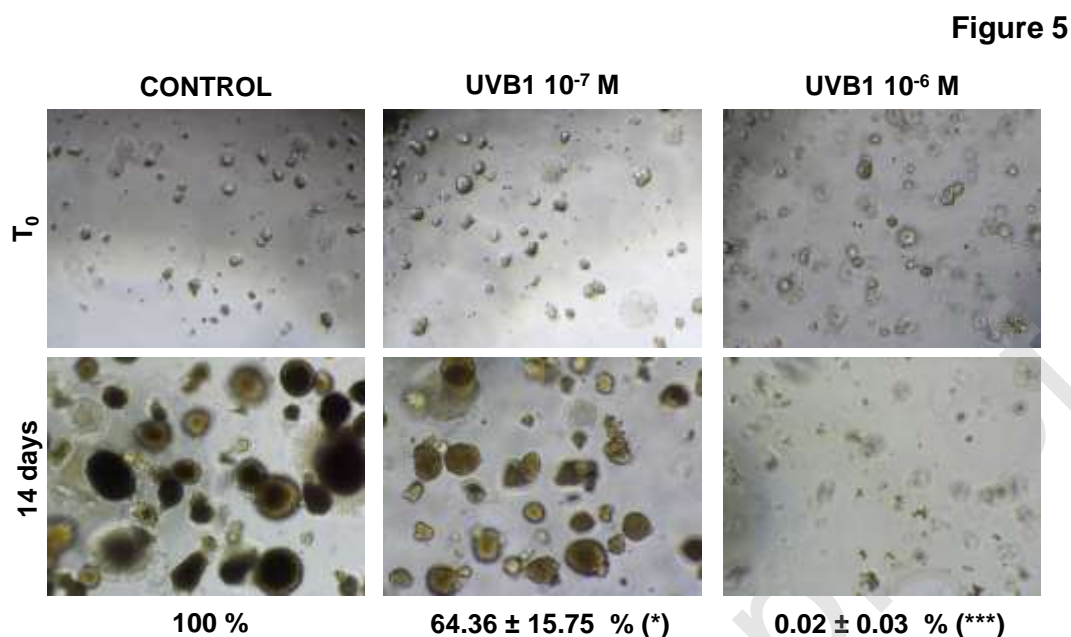| 100 % | 64.36 ± 15.75 % (*) | 0.02 ± 0.03 % (***) |

**Figure 5. UVB1 inhibits the growth of established organoids of PDX118 T-DM1 resistant cells.** Organoids obtained from R200 cells were treated with vehicle or UVB1 ($10^{-7}$ and $10^{-6}$ M) during 14 days. The assay was performed in triplicate and the number of the cells were counted by FACS. The results were expressed as percentage of vehicle-treated cells. $T_0$: initial day of organoids treatment. Representative micrographs of the conditions are shown (x40). One way ANOVA and Bonferroni post-test was applied. *p<0.05; ***p<0.001.

All these results demonstrate the ability of UVB1 to inhibit both organoids formation and its growth, reinforcing the potential use of this analogue for the treatment of T-DM1 resistant BC tumors.

**Vitamin D analogues UVB1 and EM1 reduce the viability of cells derived from TNBC-PDXs.**

A significant percentage of BC patients are grouped together as TNBCs. The main treatment for them remains chemotherapy, which usually has short-lived efficacy. Hence, it is critical to develop novel therapies for these patient population. Therefore, we decided to evaluate the effects of UVB1 and EM1 on the viability of cells derived from TNBC-PDXs. We treated a panel of six PDXs with UVB1 and EM1 at the indicated concentrations ($10^{-11}$ to $10^{-6}$ M) for 120 h.

We detected that both vitamin D analogues significantly reduced viability of TNBC-PDX cells (Figure 6). Specifically, EM1 decreased the viability in three out of six TNBC-PDXs (PDX410, PDX575 and PDX549), whereas UVB1 reduced the viability of all the TNBC-PDXs tested. Collectively, these results suggest that vitamin D analogues could be a promising therapeutic option to treat not only HER2-positive and HER2-positive TDM1-resistant, but also TNBC patients.
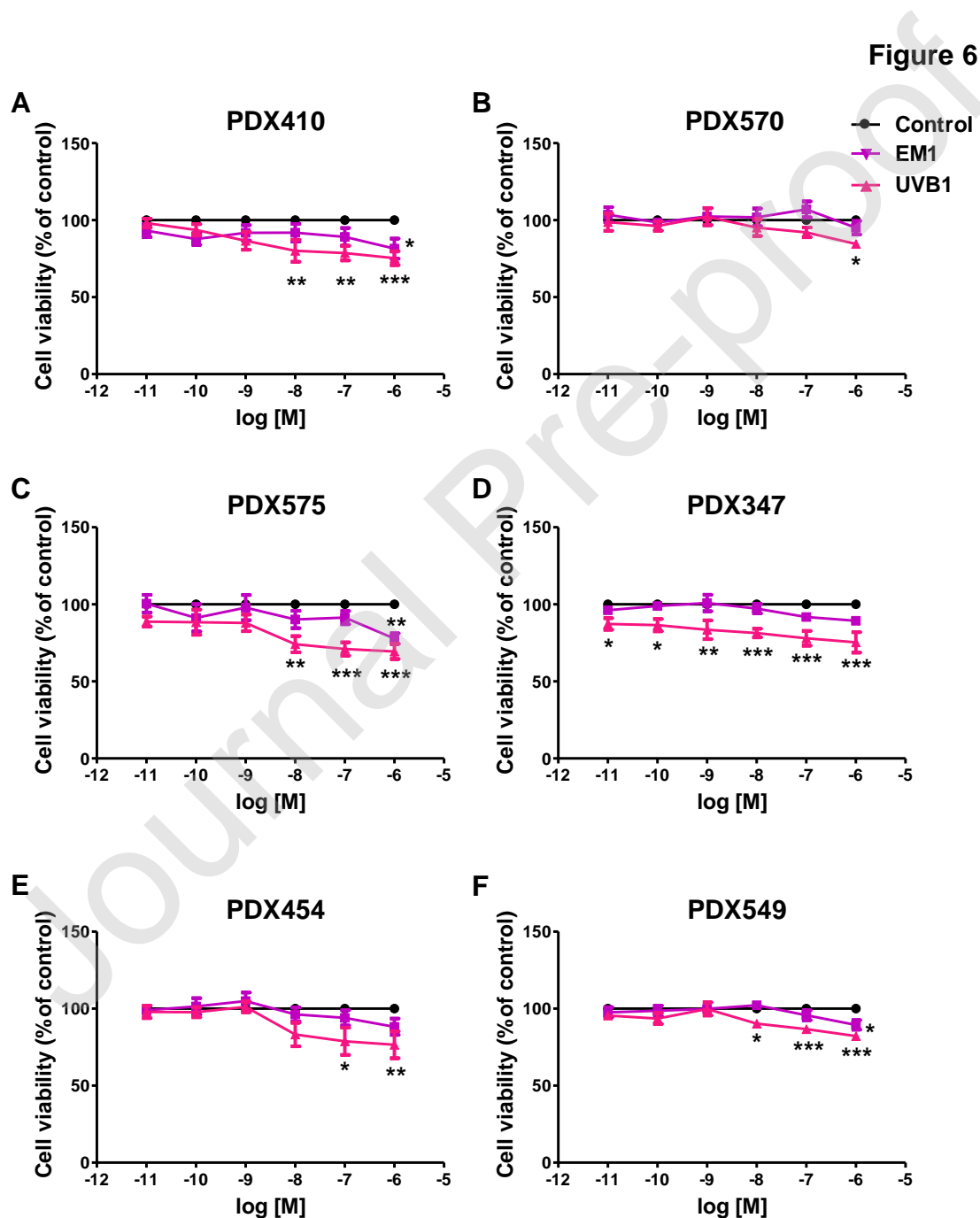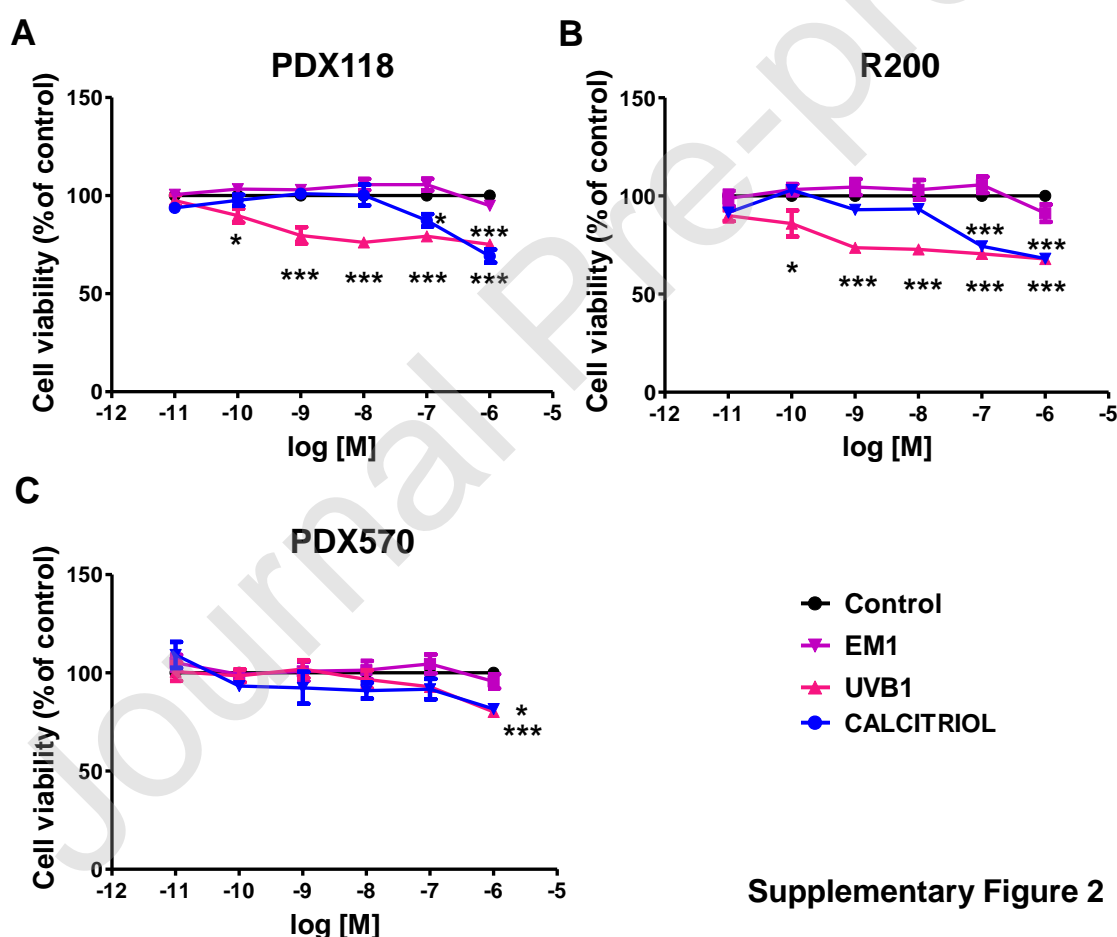


**Figure 6**

**Figure 6. Vitamin D analogues decrease the viability of cells derived from TNBC-PDXs**. Concentration-response curves obtained from cells derived from A) PDX410, B) PDX570, C) PDX575, D) PDX347, E) PDX454 and F) PDX549, exposed to vehicle, UVB1 or EM1 over a total period of 120 h and stained with crystal violet. The results were expressed as percentage of vehicle-treated cells. Data points represent mean ± SEM from at least two independent experiments. Two-way ANOVA and Bonferroni post-test was applied; *p<0.05, **p<0.01 and ***p<0.001.

Regarding the effects of the analogues compared to the natural hormone in these models, we performed viability assays including calcitriol as a positive control. We found that UVB1 was more potent than calcitriol in reducing the viability of HER2-positive PDX118, in both parental and resistant cells, and it displayed similar effect to calcitriol in the TNBC PDX570 (Supplementary Figure 2).



Supplementary Figure 2

**Supplementary Figure 2. UVB1 is more potent than calcitriol in reduce viability of cells derived from PDXs**. Concentration-response curves obtained from A) PDX118, B) R200 and C) PDX570 cells exposed to vehicle, UVB1, EM1 or calcitriol for 120 h and stained with crystal

violet. The results were expressed as percentage of vehicle-treated cells. Data points represent mean ± SEM from three independent experiments. Two-way ANOVA and Bonferroni post-test was applied; *p<0.05 and ***p<0.001.

**Relationship between analogues response and VDR expression**

Taking into account that calcitriol exerts its antitumor effects through VDR, we determined the VDR expression in PDXs used in this work to evaluate its correlation with the analogues antitumor response. As shown in Figure 7, VDR was expressed in all PDXs (Figure A and B), and a strong negative correlation was obtained between VDR expression and LogIC$_{50}$ of UVB1 (r= -0.8985, p= 0.001, Figure 7C). This means a direct correlation between VDR expression and response to UVB1. In fact, PDX570, the tumor that expresses the lowest protein level of VDR (Figure 7B), showed a very limited sensibility to UVB1 (Figure 6B). No correlation was found for EM1 analogue. These data support that the antineoplastic effectivity of UVB1 is associated with VDR protein levels.

In addition to this, we evaluated the transcription of two VDR target genes by the analogues or calcitriol (employed as a positive control) in PDX118, R200 and PDX570. As shown in Figure 7D, UVB1 significantly upregulated the transcription of *CYP24A1* gene, being this upregulation stronger in PDX118 and R200 than in PDX570. Also, UVB1 increased VDR mRNA levels in HER2-positive cells (Figure 7E). Thus, the activation of VDR by UVB1 was higher in HER2-positive PDXs that exhibit high VDR expression than in TNBC PDX that expresses the lowest VDR protein level.
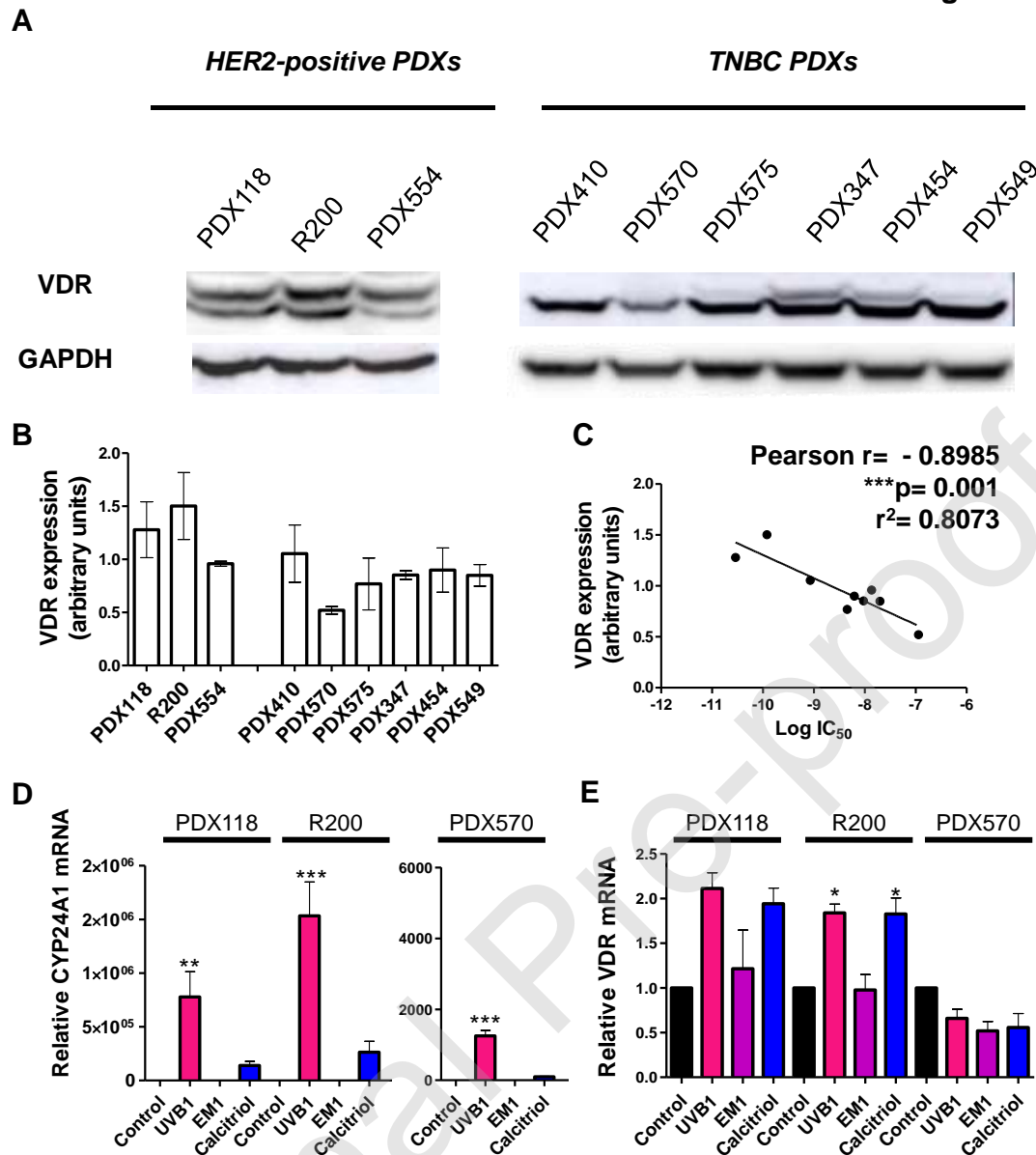
**Figure 7. VDR expression in HER2-positive and TNBC PDXs and its correlation with UVB1 antitumor response.** A) VDR expression in cells derived from different PDXs analyzed by western blot. B) The graph shows the quantification of VDR expression. Each bar represents mean ± SEM from two independent experiments. C) The graph depicts the correlation between VDR expression and LogIC$_{50}$ of UVB1 analyzed by Pearson's test; ***p<0.001. D) Relative CYP24A1 mRNA expression and E) Relative VDR mRNA expression were determined by RT-PCR in PDXs treated with vehicle, UVB1, EM1 and calcitriol (positive control) at $10^{-7}$ M during 96 h. mRNA expression was normalized to GAPDH mRNA and expressed relative to vehicle-treated cells. Each bar represents mean ± SD from three independent determinations. One way ANOVA and Bonferroni post-test was applied; *p<0.05, **p<0.01, ***p < 0.001.

## 4. DISCUSSION

In the present study we evaluated the effects of the non-hypercalcemic analogues UVB1 and EM1 on cells derived from PDXs, which are considered a powerful resource to test novel therapeutics options [22]. We demonstrated that the analogues display anti-cancer effects not only on conventional cell lines but also on primary tumor cells derived from PDXs. Importantly, we showed for the first time that UVB1 is effective to reduce the viability of resistant cells to T-DM1, which is the second line of treatment clinically employed in HER2-positive metastatic BC [23]. Thus, this analogue could be an alternative therapeutic option for tumors that are initially resistant to chemotherapies or acquire it during treatment.

As known, antitumor actions of calcitriol in different tissues, including breast tissue, are mediated by VDR [24] as it was demonstrated employing mammary tumor cells derived from VDR null mice [25]. In this study, we found that all cells derived from PDXs express this receptor. Moreover, despite of both compounds were effective in reducing cell viability of BC cells, there are differences between their antineoplastic actions. UVB1 was more potent than EM1 in that it inhibited the proliferation of both T-DM1 resistant HER2-positive and all tested TNBC cells. These differences could be explained by their binding affinities to VDR and, as a consequence, the activation of the receptor. We have previously demonstrated that both analogues display higher binding affinity to VDR than calcitriol [15,16] and, if we compare the total interaction energies of both analogues for VDR, UVB1-energy is higher than EM1-energy, demonstrating a higher UVB1-affinity to VDR. Taking these observations into account and the strong correlation between UVB1 antitumor effects and VDR expression as well as the upregulation of the VDR-target genes by UVB1 found in this work, UVB1 could be more effective against tumors that express relatively low levels of VDR. This is interesting in the context that it was recently described that low VDR expression in breast tumor tissues is associated with more aggressive characteristics [26]. Thus, UVB1 can be considered as a new therapeutic option not only for chemotherapeutic resistant tumors but also for TNBC tumors that express low levels of VDR.

Previously, we have shown that EM1 displays anti-proliferative and anti-migratory effects in glioma cells, and these actions were dependent on the presence of VDR [12,16]. In this work, the correlation between VDR expression

of PDXs and antitumor response of EM1 was not found. This lack of correlation could be due to the small amount of $IC_{50}$ employed to the test since the analogue showed antitumor effects in 4 of the 9 PDXs used in this study.

In addition to inter-tumor heterogeneity (between tumors across the patient population), intra-tumoral heterogeneity (between cells within a single tumor, and between a primary tumor and its associated metastases in a single patient) makes BC a heterogeneous collection of diseases that are difficult to treat [27,28]. Furthermore, the scenario can become more complex with the appearance of resistance to treatment. In this context, to our knowledge, this is the first work that studies the antitumor activity of calcitriol analogues employing PDXs and organoids, which are systems that retain the cellular heterogeneity present in BC. EM1 and UVB1 showed antineoplastic effects on established cancer cell lines and, also, such effects were maintained in these systems which retain a remarkable degree of biological, histological, genomic, transcriptomic, and biomarker fidelity with their tumors of origin [29]. It is noteworthy that the anti-proliferative effects of UVB1 found under 2D culture conditions were corroborated under 3D in vitro culture conditions by inhibiting the formation of organoids as well as the growth of established organoids. This suggests that the analogue could be used in different stages of BC treatment.

In summary, our results propose new therapeutic agents for tumors that are difficult to treat, such as those resistant to anti-HER2 therapies and TNBC tumors.


**DECLARATION OF INTEREST**

The authors declare no conflicts of interest.


**AUTHOR CONTRIBUTIONS**

Conceptualization, F.M.J., F.M.M. and C.A.C.; Formal analysis, F.M.J., N.S.M. and A.L.E.J.; Funding acquisition, A.J., M.M.F. and C.A.C.; Investigation, F.M.J., N.S.M., A.L.E.J., B.M.C., P.G., M.S.A.A. and M.M.; Methodology, F.M.J., N.S.M. and A.L.E.J.; Project administration, F.M.J.; Resources, B.M.C., V.C. and F.Y.; Supervision, F.M.J.; Validation, N.S.M. and

A.L.E.J; Writing - original draft, F.M.J., N.S.M. and F.M.M; Writing—review & editing, A.L.E.J., A.J. and C.A.C. All authors approved the final version of the manuscript.

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