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*Article*

# Antitumoral effects of dovitinib in triple-negative breast cancer are synergized by calcitriol *in vivo* and *in vitro*

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**Simple Summary:** The triple-negative breast cancer (TNBC) phenotype lacks a targeted therapy, is considered highly aggressive and with poor prognosis. Few therapeutic options are available for TNBC management, including chemotherapy; however, patients may develop significant adverse events and treatment-resistance. Thus, improving anticancer drugs' efficacy while reducing undesirable effects are key targets of current research. Drug combination strategies have shown to be a good therapeutic approach to address this issue, by allowing dose reduction and decreased toxicity. Regarding this, herein we explored whether combining dovitinib, a multi-kinase inhibitor, with calcitriol, a natural vitamin D anticancer metabolite, would enhance their antineoplastic effect while requiring less chemotherapeutic compound in a preclinical model of TNBC. We found that calcitriol synergized dovitinib anticancer activity *in vitro* and *in vivo*, allowing for a significant dovitinib dose-reduction while maintaining its antiproliferative potency. Our results may help to conceptualize further studies to provide TNBC-patients less toxic therapeutic options.

**Abstract:** Chemotherapy is a standard therapeutic option for triple-negative breast cancer (TNBC); however, its effectiveness is often compromised by drug-related toxicity and resistance development. Herein, we aimed to evaluate whether an improved antineoplastic effect could be achieved *in vitro* and *in vivo* in TNBC by combining dovitinib, a multi-kinase inhibitor, with calcitriol, a natural anticancer hormone. *In vitro*, cell proliferation and cell-cycle distribution were studied by sulforhodamine B-assays and flow cytometry. *In vivo*, dovitinib/calcitriol effects on tumor growth, angiogenesis and endothelium activation were evaluated in xenografted mice by caliper measures, Itgb3-immunohistochemistry and $^{99m}$Tc-RGD$_2$-tumor uptake. The drug combination elicited a synergistically improved antiproliferative effect in TNBC-derived cells, which allowed a 7-fold dovitinib dose-reduction. Mechanistically, the co-treatment induced cell death and accumulation in S and G2/M phases, while inhibited tumor growth to a greater extent than each compound alone. Tumor uptake of $^{99m}$Tc-RGD$_2$ was reduced by dovitinib, suggesting angiogenesis inhibition, which was corroborated by decreased endothelial cell growth and tumor-vessel density. In summary, calcitriol synergized dovitinib anticancer effects *in vitro* and *in vivo*, allowing for a significant dose-reduction

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of dovitinib, while maintaining its antiproliferative potency. Our results suggest the beneficial convergence of independent antitumor mechanisms of dovitinib and calcitriol to inhibit TNBC-tumor growth.

**Keywords:** breast cancer; dovitinib; calcitriol; combination index; dose-reduction index; synergism.

## 1. Introduction

Breast cancer is the most commonly diagnosed neoplasm and the leading cause of cancer death among women worldwide [1]. According to the tumor molecular expression profile, this neoplasm has been classified mainly into four different subtypes: Luminal A, luminal B, human epidermal growth factor receptor 2 (HER-2) enriched and triple-negative (TNBC) [2]. Identifying breast cancer subtypes led to personalized treatment, including endocrine and anti-HER-2 therapy; however, the therapeutic possibilities for the TNBC-subtype are limited due to the lack of specific targets. In this regard, options targeting different tyrosine kinase receptors (RTKs) are currently underway, like those directed to members of the fibroblast growth factor receptors (FGFRs) [3]. Indeed, abnormal FGFR signaling has been reported in TNBC-tumors [4], including overamplifications that may result in FGF addiction [4-7]. Blocking the FGFR pathway *in vivo* has the additional benefit of reducing tumor angiogenesis, due to its involvement in endothelial activation [8]. Regarding this, dovitinib, a potent orally bioavailable RTK inhibitor (RTKI), blocks not only FGFR 1-3, but also the vascular endothelial growth factor receptor (VEGFR 1-3) and the platelet-derived growth factor receptor (PDGFR), whose signaling pathways are involved in carcinogenesis, neovascularization, invasion, and metastasis [7]. Of note, dovitinib has been shown to inhibit FGFR, VEGFR, and PDGFR in preclinical breast cancer models [9,10]. Although dovitinib has been generally associated with low-grade side effects such as diarrhea, nausea, vomiting, and/or headache [11], its long-term use may result in more severe adverse events and/or acquired resistance [12-14]. Concerning this, a good strategy to avoid treatment-associated toxicity and resistance is to combine dovitinib with other antineoplastic agents to block additional tumor survival pathways, allowing to reduce the dose and/or frequency of administration. In this regard, a recent RNA-sequencing data analysis undertaken to identify potential targeting therapeutic candidates for TNBC, revealed that one of the highly expressed genes in these tumors was the vitamin D receptor (VDR), encoding the target of calcitriol [15]. Calcitriol, the vitamin D most active metabolite, exert potent antineoplastic activity by modulating diverse signaling networks involved in inhibition of cell proliferation, anti-inflammatory effects, acquisition of a more differentiated phenotype and induction of apoptosis [16]. Moreover, calcitriol has been shown to increase the sensitivity of tumor cells to various chemotherapeutic agents [17-21], with the added benefit of being a natural compound derived from dietary sources or by sun exposure. Notably, low vitamin D serum levels have been shown to correlate with increased risk of certain neoplasms, including breast cancer [22]. Therefore, herein we explored whether an improved *in vitro* and *in vivo* antineoplastic effect could be achieved in TNBC by combining dovitinib with calcitriol. We also used a vascular endothelial cell line as a control for endothelial activation.

## 2. Materials and Methods

### *2.1. Cell culture*

In this study, we used the TNBC cell line MBCDF-Tum (MBCDF-T) [23], and the human endothelial cell line EA.hy926 (ATCC CRL-292, Manassas VA). The cells were main-

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tained under standard cell culture conditions. All experimental procedures were performed in DMEM-F12 medium supplemented with 100 units/mL penicillin plus 100 µg/mL streptomycin and 5% charcoal-stripped-heat-inactivated fetal bovine serum.

*2.2. Proliferation studies*

Cells were seeded in 96-well plates (500-1000 cells/well) and the day after treated with dovitinib (0.005 – 5.0 µM, Santa Cruz Biotechnology, Santa Cruz, CA), calcitriol (0.1 – 100 nM, Sigma-Aldrich, St Louis, MO) or their respective vehicles (water or ethanol 0.1%) for 6 days. Afterward, cell proliferation was evaluated by the sulforhodamine B colorimetric assay, as previously described [24]. Absorbance was read at 492 nm in a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek, VT, USA). The concentration values that inhibited cell proliferation at 20% ($IC_{20}$) and 50% ($IC_{50}$) were calculated by the dose-response fitting function, using the scientific plotting software Origin 9.0 (OriginLab Corporation, Northampton, MA, USA).

*2.3. Combination index and dose reduction index determination*

To identify the nature of the compounds combination effect, the combination index (CI) and dose reduction index (DRI) were calculated as previously reported [25-27]. Results were evaluated considering that CI values < 1, = 1 or >1 depict synergistic, additive, or antagonistic effects, respectively, while synergism is subdivided into nearly additive (0.90–1.10), slight synergism (0.85–0.90), moderate synergism (0.7–0.85), synergism (0.3–0.7), strong synergism (0.1–0.3), and very strong synergism (<0.1) [27]. On the other hand, DRI values ≤ 1 or >1 indicate not favorable dose-reduction or favorable dose-reduction, respectively [27].

*2.4. Cell cycle analysis*

Flow cytometry analyses were performed using a FACS Aria II flow cytometer (Becton Dickinson, San Jose, CA, USA). Briefly, MBCDF-T cells were treated with dovitinib ($IC_{50}$) and/or calcitriol (10 nM) for 72 h. After that, the cells were harvested, washed in PBS pH 7.2, fixed in 70% ethanol, and kept at -20° C until analysis. DNA staining with 7-amino-actinomycin D (BioLegend, San Diego, CA) was done as reported previously [23]. The results were analyzed using FlowJo Software (LLC, Ashland, OR, USA).

*2.5. Induction of tumors in athymic nude mice and therapeutic protocol*

Six-week-old female athymic nude mice (BALB/c homozygous, Crl:NU(NCr)-Foxn1nu, Charles River Laboratories, Wilmington, MA) were maintained under controlled temperature, humidity and 12 h light/dark cycles with sterile food (standard PMI 5053) and water ad libitum. Mice were randomly divided into four experimental groups (N = 4 each): 1) Control (C, 100 µL sterile saline 0.9% NaCl i.p. q.wk), 2) Calcitriol (Cal, 0.25 µg/ 100 µL i.p. q.wk, Geldex, GELpharma, México), 3) Dovitinib (Dov, 20 mg/kg twice a week i.p, CAS 852433-84-2, Santa Cruz), and 4) Dovitinib plus calcitriol (Dov+Cal). The treatments were initiated the next day after the subcutaneous injection of MBCDF-T cells (1.0 x 106 / 0.1 mL sterile 0.9% NaCl) into the back of mice and were maintained for 3 weeks. To determine any toxic effect of the drug, mice were weighed three times per week. Tumor volume was calculated by caliper measures and the standard formula (length x width2)/2, where length and width are the largest and smallest dimension, respectively. At the end of the experiments, mice were sacrificed by cervical dislocation under anesthesia (sodium pentobarbital 80 mg/kg i.p.), and tumors were fixed in paraformaldehyde for immunohistochemical staining.

*2.6. SPECT/CT imaging*

To acquire tumor images of activated endothelium, one mouse from each group was placed in the prone position in an induction chamber and anesthetized (2% isoflurane in 100% oxygen). Under anesthesia, an intravenous injection of $^{99m}$Tc-RGD$_2$ (7.4 MBq/ 0.05 mL, ININ, México), a marker of endothelial activation, was administered. After 3-

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4 h, the radiopharmaceutical tumor uptake was evaluated using a micro single-photon emission computed tomography and radiographic computed tomography (SPECT/CT) scanner (Albira, ONCOVISION; Gem Imaging S.A., Valencia, Spain). Acquisition parameters were the same as reported previously [23].

*2.7. Itgb3 immunohistochemistry and tumor vessel density evaluation*

To visualize blood vessels, formaldehyde-fixed and paraffin-embedded tumor sections placed on glass coverslips were dewaxed and rehydrated using standard protocols. Antigen retrieval was accomplished by autoclaving in retriever citrate solution (BioSB, Santa Barbara, CA, USA). Tumor slides were blocked with immunodetector peroxidase blocker (BioSB) and incubated for 1 h with the primary antibody anti-Integrin 3 (Itgb3 1:100, Cell Signaling Technology, Beverly, MA 13166, USA), a marker of activated endothelium. After washing, slides were sequentially incubated with Immuno-Detector Biotin-Link and Immuno-Detector HRP label (BioSB) 10 min each. Staining was completed with diaminobenzidine and slides were counterstained with hematoxylin. Images were taken with a conventional microscope. Microvessel count was undertaken by three different observers using 20X photographs and considering Itgb3-positive vessels in at least three hot spots areas of each tumor, as described previously [28].

*2.8. Statistical analysis*

Statistical differences were established by one-way ANOVA followed by appropriate post-hoc tests for multiple comparisons using a specialized software package (SigmaStat 3.5, Jandel Scientific, CA, USA). Differences were considered statistically significant at $P < 0.05$.

## 3. Results

*3.1. Dovitinib and calcitriol differentially regulate breast cancer and endothelial cell proliferation*

The effects of dovitinib and calcitriol on MBCDF-T and EA.hy926 cell proliferation are shown in Figure 1. As depicted, dovitinib significantly inhibited breast cancer and endothelial cell proliferation in a concentration-dependent manner (Figures 1A and 1B). On the other hand, as expected and as previously reported [23], calcitriol only inhibited MBCDF-T cell proliferation and did not affect endothelial cells growth (Figure 1C and 1D, respectively).

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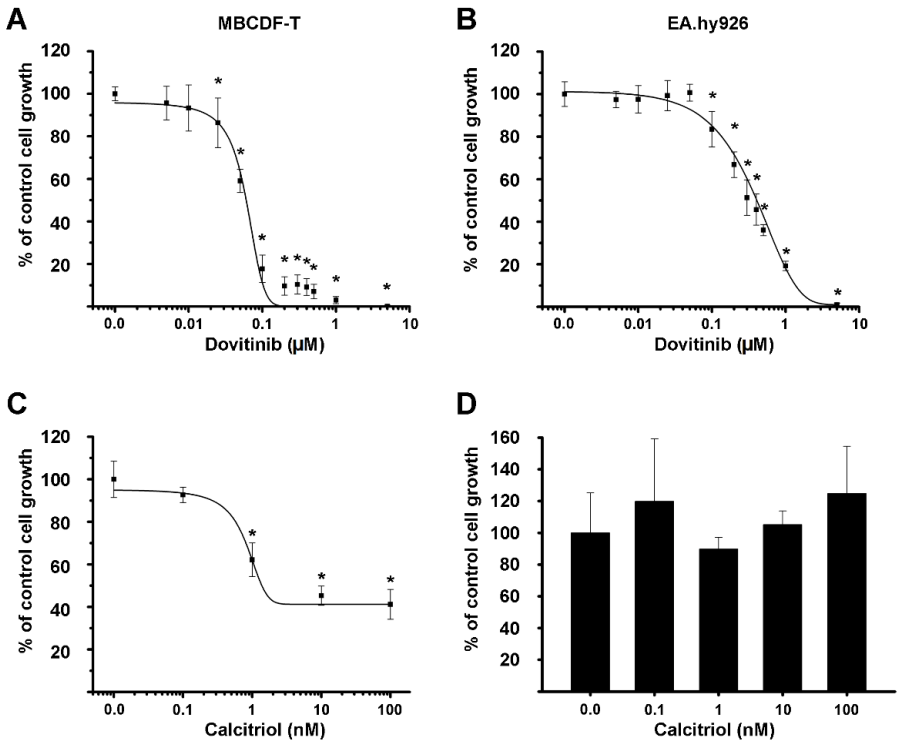


**Figure 1.** Dose-response curves of dovitinib and calcitriol in MBCDF-T and EA.hy926 cells. Cancer (A, C) and endothelial (B, D) cells were incubated with dovitinib (A, B) or calcitriol (C, D) for 6 days. The results are depicted as the mean ± S.D. of at least three independent experiments by sextuplicate and were normalized vs. control values, which were set to 100%. * P<0.001 vs. control.

Based on the dose-response curves, $IC_{20}$ and $IC_{50}$ values were calculated (Table 1) and were taken into account for the combined treatments. Considering dovitinib $IC_{50}$ values, this drug inhibited more potently MBCDF-T cell growth than EA.hy926 (Table 1).

**Table 1**. $IC_{20}$ and $IC_{50}$ values of dovitinib and calcitriol in endothelial and TNBC-cells proliferation

| Cell line | Dovitinib (nM) | | Calcitriol (nM) | |
|---|---|---|---|---|
| | $IC_{20}$ | $IC_{50}$ | $IC_{20}$ | $IC_{50}$ |
| **MBCDF-T** | 18 | 56 | 0.296 | 0.619 |
| **EA.hy926** | 153 | 378 | ND | ND |

Inhibitory concentrations at 20% ($IC_{20}$) and 50% ($IC_{50}$) were calculated based on the dose-response curves of dovitinib and calcitriol. Results are depicted as the mean of N ≥ 3 experiments. The ICs of calcitriol in EA.hy926 cells were not determined due to the lack of anti-proliferative effect of this compound in these cells (ND = Not determined).

*3.2. The combination of dovitinib and calcitriol synergistically inhibited the growth of TNBC-cells*

Next, we sought to determine the nature of the pharmacological interaction between dovitinib and calcitriol in MBCDF-T cells. For this, the following combination schemes were evaluated (dovitinib:calcitriol): $IC_{20}$:$IC_{20}$, $IC_{20}$:$IC_{50}$, $IC_{50}$:$IC_{20}$ and $IC_{50}$:$IC_{50}$. With all these combinations, a significantly stronger cell growth inhibitory effect was achieved than that obtained with each drug alone (Figure 2A). Notably, the calculated

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CI values showed that this effect was synergic (CI < 1) in all cases (Table 2). Then, we studied the combined effect of both drugs in EA.hy926 cells. Since calcitriol did not change endothelial growth, we tested dovitinib $IC_{20}$ and $IC_{50}$ values with 0.1 and 10 nM of calcitriol. As seen in Figure 2B, calcitriol did not change dovitinib potency to inhibit endothelial cells proliferation.

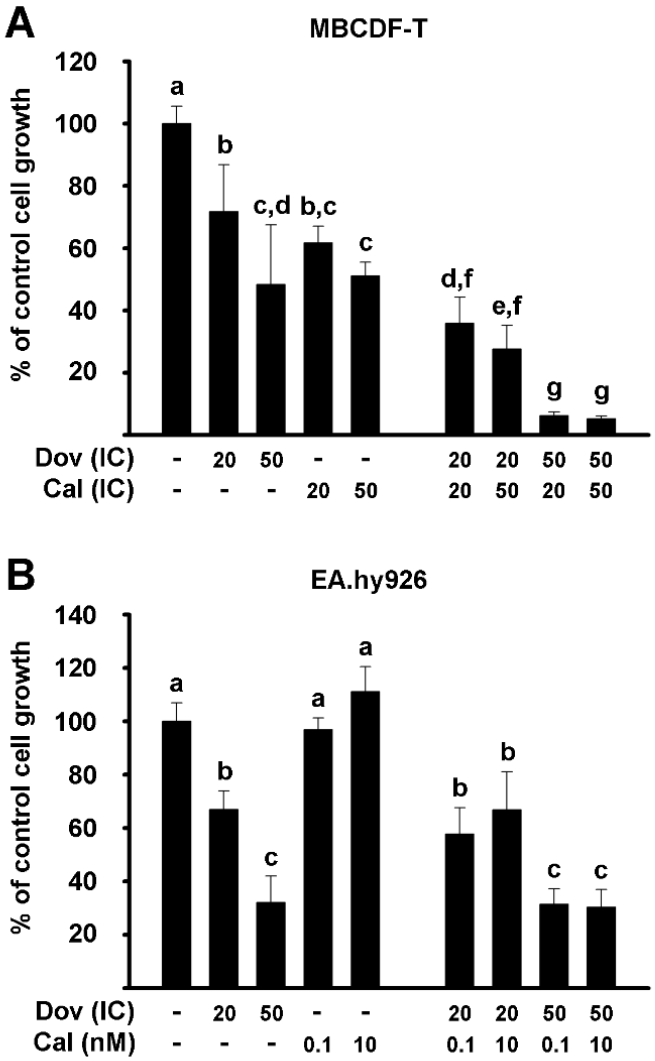


**Figure 2.** Calcitriol synergizes dovitinib antiproliferative activity in MBCDF-T cells. MBCDF-T cells (A) and EA.hy926 cells (B) were incubated in the presence of dovitinib (Dov), calcitriol (Cal) or their combination at their respective inhibitory concentration (IC) values at 20% or 50% during 6 days. For EA.hy926 cells (B), Dov $IC_{20}$ and $IC_{50}$ were combined with Cal 0.1nM and 10 nM. Each bar represents the mean ± S.D. of at least four independent experiments by triplicate and were normalized vs. control values, which were set to 100%. Different letters indicate statistical significance ($P < 0.05$).

*3.3. The combination of dovitinib with calcitriol allows for a significant dovitinib-dose reduction*

Considering the synergism elicited by the combination of dovitinib with calcitriol in tumor cells, we calculated the DRI values to determine how many folds the concentration of each compound could be reduced while maintaining the same efficacy as the drug alone (Table 2). Remarkably, in all combination schemes both dovitinib and calcitriol concentrations showed favorable DRI values (DRI > 1), in accordance with the intensity of synergism (Table 2). As depicted in this table, the most favorable combination schemes were $IC_{50}$:$IC_{50}$ and $IC_{50}$:$IC_{20}$ for dovitinib:calcitriol, since dovitinib may be

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reduced up to 7 folds while calcitriol up to 28 folds, respectively. Taken together, these data show that the combination of dovitinib and calcitriol is synergistic, with favorable dose reduction values.

**Table 2.** Combination index values and dose reduction index for dovitinib:calcitriol treatment of MBCDF-T cells

| Combination schemes | CI | Description | DRI (folds) | |
|---|---|---|---|---|
| Dov:Cal | | | Dov | Cal |
| $IC_{20}$:$IC_{20}$ | 0.491 | Synergism | 4.58 | 3.66 |
| $IC_{20}$:$IC_{50}$ | 0.564 | Synergism | 5.92 | 2.53 |
| $IC_{50}$:$IC_{20}$ | 0.197 | Strong synergism | 6.18 | **28.62** |
| $IC_{50}$:$IC_{50}$ | 0.203 | Strong synergism | **7.04** | 16.50 |

The combination index values (CI) and dose reduction index (DRI) were calculated after co-incubating MBCDF-T cells in the presence of the inhibitory concentrations at 20% ($IC_{20}$) and 50% ($IC_{50}$) of dovitinib (Dov) and calcitriol (Cal). CI <1, =1, and >1 indicate synergistic, additive, or antagonistic effects, respectively. In the synergy scale, values <0.1, 0.1-0.3, 0.3-0.7, 0.7-0.85, 0.85-0.90, and 0.90–1.10 indicate very strong synergism, strong synergism, synergism, moderate synergism, slight synergism, and nearly additive, respectively. DRI values = 1, >1, and <1 indicate no dose-reduction, favorable dose-reduction or not favorable dose-reduction, respectively, for each drug in combination. DRI values higher than 1 represent the folds of dose reduction that is allowed in combination for a given degree of effect compared with the dose of each drug alone.

### *3.4. The combination of dovitinib and calcitriol promoted breast cancer cell death*

To gain insight into the mechanisms associated with the synergic antiproliferative effect of calcitriol and dovitinib, we studied the cell cycle distribution of MBCDF-T cells when exposed to both compounds alone and combined. As depicted in Figure 3, dovitinib, calcitriol and their combination significantly increased the percentage of cells in S-phase with a concomitant reduction in the percentage of cells in the G1-phase. Notably, dovitinib alone induced cell death (Sub-G1) compared to the control; however, its combination with calcitriol significantly increased this parameter. Also, the drug combination slightly but significantly augmented the percentage of cells in G2/M phases of the cell cycle (Figure 3).

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**Percentage of cells in each cell cycle-phase**

| Treatment | SubG1 | G0/G1 | S | G2/M |
|---|---|---|---|---|
| Control | 1.32 ± 1.13[a] | 66.13 ± 4.87[a] | 29.20 ± 5.10[a] | 1.32 ± 0.43[a] |
| Dov | **5.65 ± 1.46[b]** | **44.70 ± 4.67[b]** | **47.23 ± 3.56[b]** | 1.87 ± 1.15[a] |
| Cal | 0.90 ± 0.64[a] | **52.53 ± 5.13[b]** | **45.50 ± 5.51[b]** | 1.54 ± 0.47[a] |
| Dov + Cal | **21.23 ± 2.70[c]** | **27.13 ± 6.71[c]** | **49.97 ± 11.74[b]** | **4.57 ± 0.95[b]** |

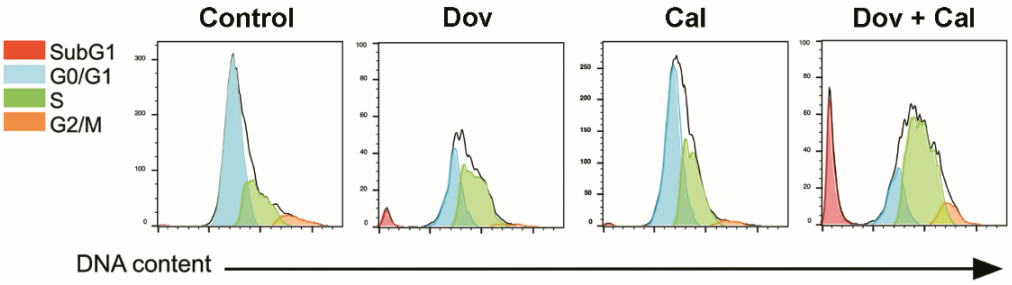


**Figure 3.** Modification of cell cycle distribution by dovitinib, calcitriol, and their combination in TNBC MBCDF-T cells. The effects of dovitinib (Dov, $IC_{50}$), calcitriol (Cal, 10 nM), and their combination (Dov + Cal) on cell cycle distribution were evaluated in MBCDF-T cells. Results are shown as the mean ± S.D. of three independent experiments. Different letters indicate statistical significance ($P < 0.05$). Representative flow cytometry plots are shown in the lower panel. Cells in G1-peak are shown in blue, whereas S-region cells are shown in green and G2/M cells in orange. SubG1 subpopulation, corresponding to dead cells, is shown in red.

*3.5. In vivo co-administration of dovitinib and calcitriol significantly decreased tumor volume in a greater extent than each compound alone.*

Based on the effective antiproliferative action of calcitriol + dovitinib combination observed *in vitro*, we decided to evaluate the antitumor effect of this scheme in a murine model *in vivo*. As expected, and as previously shown for calcitriol [23], this compound and dovitinib *per se* slowed MBCDF-T tumor growth compared to the control group. However, the co-administration of these compounds significantly reduced tumor volume to a greater extent than each compound alone (Figure 4A). Of note, there were no apparent side effects induced by the treatments, as judged by the absence of diarrhea and weight loss, suggesting no treatment-associated toxicity at the doses tested.

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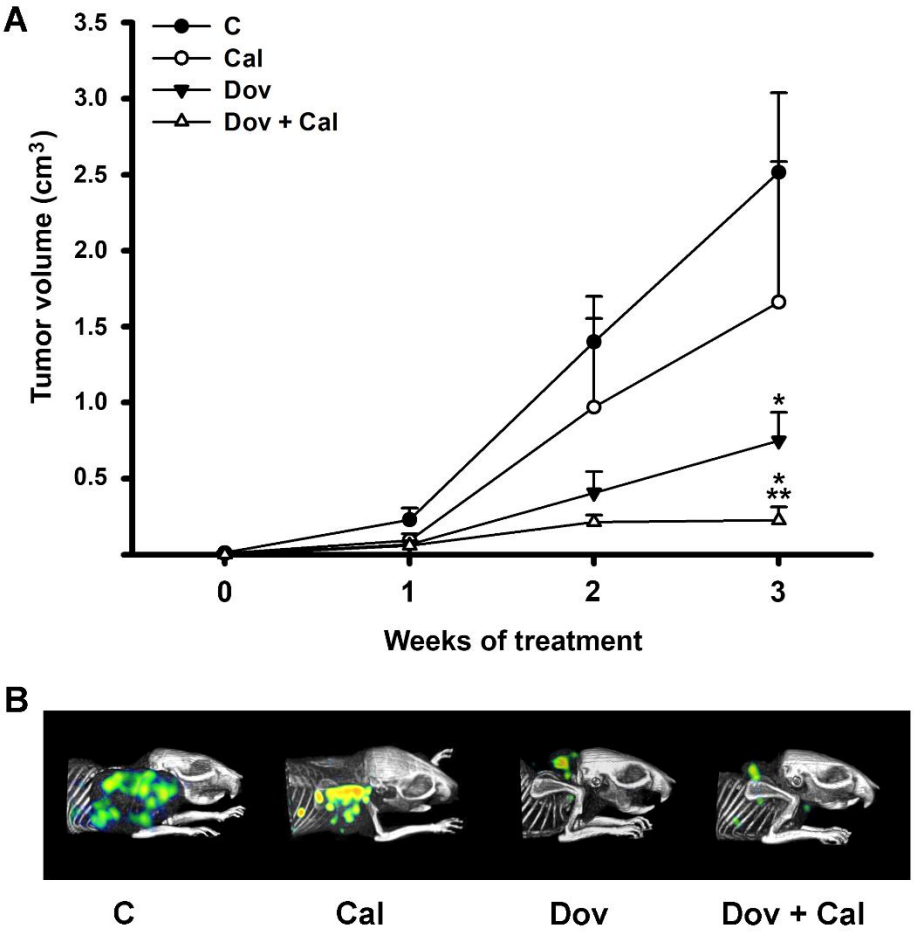


**Figure 4.** *In vivo* antitumor and antiangiogenic effects of dovitinib in combination with calcitriol. MBCDF-T cells were xenografted in female nude mice, starting treatments the following day with saline solution (C), calcitriol (Cal), dovitinib (Dov), or their combination (Dov + Cal) during three weeks. During the experiment, tumor volume was calculated, and the results are depicted as the mean ± SEM (A). N = 4 mice per treatment. * $P < 0.05$ vs. C, ** $P < 0.05$ vs. Dov and Cal. At the end of the treatments, $^{99m}$Tc-RGD$_2$ tumor uptake was evaluated in representative mice by SPECT/CT imaging (B).

### *3.6. The in vivo antiangiogenic activity of dovitinib was not affected by calcitriol*

In a representative mouse from each group, SPECT/CT images were acquired at the end of the experiment, showing decreased $^{99m}$Tc-RGD$_2$ tumor uptake in treated mice, with a greater reduction in dovitinib and dovitinib+calcitriol groups (Figure 4B). Then, to quantitatively assess the effect of the treatments in tumor angiogenesis, vessel count was performed in Itgb3-immunostained slides. We knew from previous studies [23,29] that calcitriol does not modify tumor angiogenesis in breast tumor xenografts and that its administration may increase vascular endothelial growth factor (VEGF) and FGF levels [29-31]. Therefore, we expected that the use of calcitriol with an antiangiogenic factor such as dovitinib would improve overall anticancer properties. However, it was necessary to ascertain that dovitinib antiangiogenic activity prevailed in the presence of calcitriol. As seen in Figure 5, tumors in dovitinib-treated mice significantly had a fewer number of vessels, as compared to controls. This effect was preserved in the tumors of mice co-treated with dovitinib and calcitriol (Figure 5).

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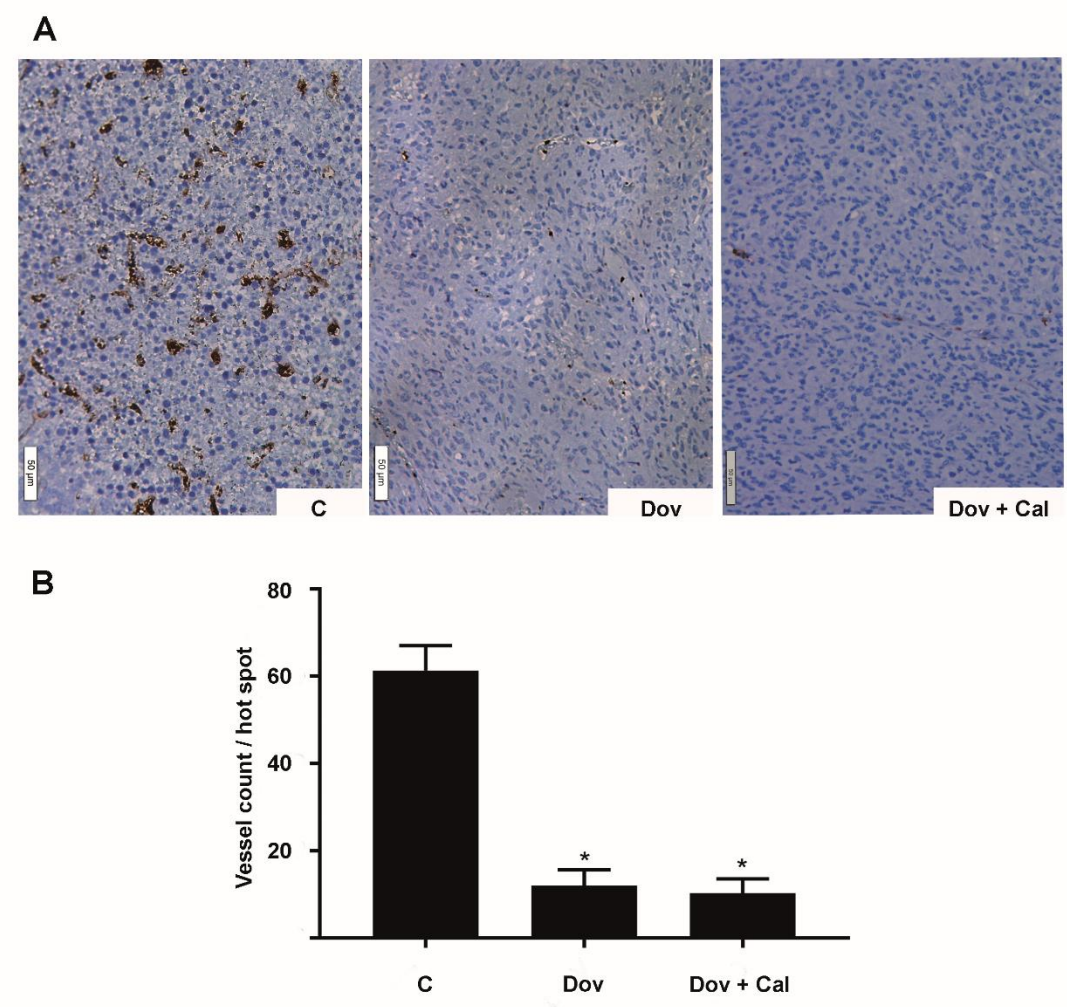


**Figure 5.** Vessel density was analyzed in tumors from control mice (C), treated with dovitinib (Dov), or with the combination of dovitinib and calcitriol (Dov + Cal) by Itgb3 immunostaining. (A) Representative images show Itgb3 in brown staining with 20X magnification, scale bar indicates 50 μm. (B) Vessel count per hot spot. Results are depicted as the mean ± SEM of the number of vessels in three different hot spots per tumor (N = 4 different tumors/treatment), $P < 0.05$ vs. C.

## 4. Discussion

Due to the heterogeneous nature of TNBC, the poor prognosis, and the lack of targeted therapy, these tumors remain a clinical challenge nowadays. However, the high expression of diverse RTKs in most TNBC tumors makes these markers plausible oncological targets [32]. Drugs with multikinase inhibitory activity are a good option to treat TNBC patients, since the concomitant targeting of different RTKs translates into increased efficacy and reduced resistance. Nevertheless, some adverse side effects may develop. Here, we evaluated the *in vivo* and *in vitro* pharmacological interaction of dovitinib and calcitriol with the objective to potentiate their anticancer effect while allowing for dose reduction in TNBC. The analysis of our results using the CI theorem of Chou-Talalay [26], showed that in TNBC-cells the combination of calcitriol with dovitinib was highly synergic, reaching CI values as low as 0.2 in the $IC_{50}$:$IC_{20}$ scheme. Remarkably, in all the combination schemes, the dose-reduction for each drug was favorable (DRI > 1) and was greater when the synergism was stronger. In this sense, the greatest dose-reduction was observed with the combination of dovitinib:calcitriol $IC_{50}$:$IC_{20}$ and $IC_{50}$:$IC_{50}$, where each compound concentration can be reduced by more than 7 and 28 folds, respectively. Overall, these results suggest the possibility to reduce the compounds' dose and therefore toxicity and resistance in therapeutic applications.

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To gain mechanistic insight into the drug synergism, we evaluated the effect of the combined treatment on the cell cycle distribution, and found that it strongly induced cell dead in TNBC-cells (Sub-G1 cells). Interestingly, we also found a significant accumulation of cells in the S-phase of the cell cycle in cells treated with all compounds alone and combined. Accumulation of cells in the S-phase may suggest DNA damage or inhibition of the DNA-replication machinery. Indeed, some RTKIs may directly interact with the DNA, inhibiting cancer cell proliferation [33]. Particularly in the case of dovitinib, its antiproliferative activity results not only from inhibiting multiple kinases, but also, in part, from its ability to block the ATP binding site of topoisomerases [34], which are DNA-interacting enzymes essential for proliferating cells [35]. Of note, calcitriol or its analogs have shown to enhance the efficacy of topoisomerase-inhibitors in cancer cells, as well as to induce breast cancer cell apoptosis [36,37]. Moreover, the flow cytometric analysis of our data also indicated that the dovitinib-calcitriol combination significantly increased the percentage of G2/M-cells, which may suggest mitotic catastrophe, a process associated to cell death. This phenomenon has been previously demonstrated in diverse cancer cell lines, where dovitinib promoted a delay in mitotic exit, causing G2 arrest by the activation of the DNA damage checkpoint [38]. Interestingly, enriching the number of G2-phase cells has resulted in enhanced radiosensitivity [39]; therefore, future studies are warranted combining dovitinib and calcitriol with radiotherapy.

It is known from earlier studies that calcitriol may either favor or inhibit angiogenesis, by stimulating proangiogenic factors or downregulating some RTKs, including FGFR1 [23,29-31,40,41]. However, considering that this hormone inhibits breast cancer cell proliferation through different mechanisms [16,42], we hypothesized that its combination with a VEGFR/FGFR-targeting agent would improve overall anticancer effects. Our results showed that, while the antitumor effects of both compounds were synergistically enhanced on *in vivo* and *in vitro* conditions, the antiangiogenic activity of dovitinib remained unchanged in the presence of calcitriol, as shown in endothelial cell proliferation, tumor vessel count, and $^{99m}$Tc-RGD$_2$ tumor uptake. This was a positive effect that suggests the convergence of different antitumorigenic mechanisms of calcitriol and dovitinib, resulting in a beneficial anticancer outcome. Similarly, previous studies have shown that vitamin D derivatives exert synergistic effects when used in combination with other oncological drugs [18-21]. In particular, it has been demonstrated that the combination of calcitriol or its analogs with gefitinib, a synthetic RTKI, enhances global anticancer activity by inhibiting tumor growth and inducing apoptosis, and in cancer patients it does not result in serious undesirable side effects [17,20,43,44]. To the best of our knowledge, this is the first study addressing the antineoplastic effects of dovitinib in combination with calcitriol. Notably, the effective inhibitory concentrations of calcitriol determined herein and in other studies [23], which are in the nanomolar range, are significantly lower than the blood levels reached in calcitriol-treated cancer patients in which little toxicity has been reported [43,45], making it a safely achievable dose in the clinic. Likewise, the dovitinib $IC_{50}$ values calculated herein for breast cancer cells (56 nM, equivalent to 22 ng/mL), were significantly lower than the previously reported serum levels in dovitinib-treated patients (60 – 100 ng/mL) [46].

Limitations of this study include that overall survival was not evaluated as an endpoint in the *in vivo* murine model. This would have greatly improved the conclusions. Also, this study did not assess the mechanism by which tumors may generate escape routes after the antiangiogenic therapy, including vascular mimicry, which remain the subject of future studies.

## 5. Conclusions

In summary, we demonstrated herein that at clinically achievable and safe concentrations, the combination of calcitriol with dovitinib is highly synergic in inhibiting tumorigenesis and breast cancer cell proliferation. Mechanistically, the combination regimen inhibited tumor angiogenesis and induced cancer cell death as well as S and G2/M-phases arrest. The conclusions of this study suggest the feasibility for dose reduction to avoid dose-related toxicity while retaining therapeutic efficacy in a combined treatment scheme for TNBC in the clinic. Further studies are warranted to explore dovitinib and calcitriol in combination with radiotherapy.

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