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VDR Agonists Increase Sensitivity of MCF-7 and BT-474 Breast Cancer Cells to 5 FU

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Abstract. *Background/Aim: The study aimed to test the potential for increasing the antiproliferative activity of 5-fluorouracil against breast cancer cells of various molecular subtypes by vitamin D receptor (VDR) agonists, calcitriol and tacalcitol, used at a low concentration of 10 nM. Materials and Methods: Calcitriol and tacalcitol were used to increase the antiproliferative effect of 5-fluorouracil against the following human breast cancer cell lines: MCF-7, T47D, BT-474 (luminal); JIMT-1, SKBR-3 (HER2-enriched); MDA-MB-231 (triple-negative/basal-B), and non-malignant MCF-10A breast epithelial cells. Results: Both calcitriol and tacalcitol significantly increased the sensitivity of MCF-7 and BT-474 cells to the antiproliferative effect of 5-fluorouracil, while no increase in the sensitivity of MDA-MB-231 cells to 5-fluorouracil treatment was observed. Conclusion: The VDR agonist used at the relatively low concentration of 10 nM may increase the sensitivity of breast cancer cells, at least of the luminal subtype, to the antiproliferative effect of 5-fluorouracil.*

5-Fluorouracil (5-FU) is an analog of uracil, and its antiproliferative and cytotoxic effects are associated with the incorporation of its metabolites into nucleic acids, and with the inhibition of the enzymatic activity of thymidylate synthase (1). In the treatment of patients with breast cancer after surgery or with metastatic breast cancer, 5-FU is administered intravenously or 5-FU is formed inside a cancer cell from liver-activated capecitabine, an orally administered prodrug (1, 2). 5-FU is intracellularly converted into the active form capable of inhibiting thymidylate synthase (1, 3). Thymidylate synthase activity is necessary for proliferation because it catalyzes the biosynthesis of thymidylate needed for DNA replication. However, increased level of thymidylate synthase may be one of the causes of reduced sensitivity to 5-FU (1).

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily of transcription factors, which (in addition to estrogen, progesterone and androgen receptors) is expressed in normal mammary gland and in the majority of human breast cancers (4). VDR is involved in cell growth regulation, apoptosis and cell differentiation (5). The natural VDR ligand is 1,25-dihydroxyvitamin D_3 (calcitriol). Synthetic vitamin D_3 analogs that have VDR agonist activity, but with lower VDR-mediated calcemic activity, are also promising for use in anticancer therapy, one of which is 1,24-dihydroxyvitamin D_3 (tacalcitol, PRI-2191) (6, 7).

The antiproliferative activity of VDR agonists is at least partly associated with the activation of the *CDKN1A* and *CDKN1B* genes encoding p21 (WAF1/CIP1) and p27 (KIP1) proteins (8, 9), which inhibit cyclin/cyclin-dependent kinase activity and cell cycle progression through G_1-phase into S-phase. Calcitriol has been shown to downregulate *TYMS* gene encoding thymidylate synthase (10). Tacalcitol, more strongly than calcitriol, reduced the expression of the *TYMS* gene in colorectal cancer cells (11). However, the exact mechanism by which VDR agonists reduce thymidylate synthase expression is still poorly understood (11, 12). Despite the controversy over the mechanism of action, it appears that VDR agonists, by inhibiting the expression of thymidylate synthase, may potentiate the anticancer effect of 5-FU. Indeed, calcitriol and tacalcitol have been shown to potentiate 5-FU antiproliferative activity against colorectal cancer cells *in vitro* (11) and tacalcitol increased the antitumor activity *in vivo* of both capecitabine and 5-FU in mice bearing colon tumors (13). Therefore, it can be expected that VDR agonists may also potentiate the antiproliferative effect of 5-FU against breast cancer cells. It has recently been found that treatment with 100 nM of calcitriol has significantly improved the antiproliferative efficacy of 5-FU against MCF-7 cells (14). It should be noted, however, that the calcitriol concentration used was

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Table I. *The growth inhibitory effect of 5-FU against human breast cancer cells and breast epithelial cells in the absence or presence of VDR agonists, calcitriol and tacalcitol. Cells were treated for 120 h, and IC_{50} values for 5-FU were calculated based on cell viability as assessed by MTT assay. Mean IC_{50} (μM)±standard deviations for the results of four independent experiments are shown.*

Cell line	5-FU alone	5-FU with 10 nM calcitriol[a]	5-FU with 10 nM tacalcitriol[a]	IC_{50} ratio[b]
MCF-7	3.65±0.87	2.52±0.71*	1.65±0.69*	2.2
T47D	6.43±5.65	3.08±2.17	3.91±1.61	1.6
BT-474	34.00±8.30	20.47±10.41*	18.79±6.64*	1.8
JIMT-1	6.61±5.53	3.73±1.48	4.49±2.54	1.5
SKBR-3	5.74±4.04	2.92±0.83	2.94±2.20	2.0
MDA-MB-231	23.98±14.61	23.93±10.92	23.88±7.47	1.0
MCF-10A	19.28±12.49	12.07±1.57	12.37±5.52	1.6

[a]Percentages of cell growth inhibition after 120 h treatment with 10 nM VDR agonist (calcitriol/tacalcitol) used alone: 17±8/12±23 (MCF-7), 0±30/0±24 (T47D), –2±9/–1±18 (BT-474), –8±17/0±14 (JIMT-1), 23±13/23±9 (SKBR-3), –9±16/–7±10 (MDA-MB-231), –6±11/–14±24 (MCF-10A). [b]IC_{50} ratio: IC_{50} of 5-FU alone/IC_{50} of 5-FU with tacalcitol. *Significant reduction of 5-FU IC_{50} in the presence of VDR agonist compared to treatment with 5-FU alone, $p<0.05$ (one-tailed *t*-test).

quite high compared to the level of 48-156 pM in serum (15). We tried to increase the antiproliferative effect of 5-FU against cell lines representing various molecular subtypes of breast cancer (16) through the use of VDR agonists, calcitriol and tacalcitol, at a 10-fold lower concentration of 10 nM. Our results indicate a statistically significant increase in 5-FU sensitivity of two of the six breast cancer cell lines tested, namely MCF-7 and BT-474, which represent luminal subtypes of breast cancer.

Materials and Methods

Reagents. Calcitriol (1,25-dihydroxyvitamin D_3) and tacalcitol (1,24-dihydroxyvitamin D_3) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in ethanol to a concentration of 100 μM. Then, these solutions were diluted in culture medium to the required concentrations. 5-Fluorouracil at a concentration of 50 mg/ml was purchased from Accord Healthcare Poland (Warsaw, Poland) and diluted prior to use in culture medium to the required concentrations.

Cell lines. The following cell lines representing various molecular subtypes of human breast cancer were used: MCF-7 and T47D (luminal A), BT-474 (luminal B), JIMT-1 and SKBR-3 (HER2-enriched), MDA-MB-231 (triple-negative/basal-B, TNB). MCF-7 and T47D cell lines were obtained from European Collection of Authenticated Cell Cultures (ECACC, Porton Down, Salisbury, UK), JIMT-1 was obtained from DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Germany), BT-474, SKBR-3, MDA-MB-231 adenocarcinoma cell lines and non-malignant breast epithelial MCF-10A cell line were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

Western blot analysis. VDR protein levels were determined in lysates made from the same cell cultures that were harvested for MTT assay. Whole cell lysates were prepared using RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Poznan, Poland). Equal amounts of protein (40 μg) were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE). Proteins were transferred to a PVDF membrane (Immobilon-FL, Merck Millipore, Burlington, MA, USA) and incubated afterward for 1 h at room temperature in a blocking solution containing 1% non-fat dry milk in a buffer containing 50 mM Tris-HCl, pH 8.0 and 0.15 M NaCl supplemented with 0.05% Tween 20. The membrane was then incubated overnight with rabbit anti-VDR IgG antibody (catalog no. 12550, Cell Signaling Technology, Danvers, MA, USA) at 4˚C followed by incubation with secondary horseradish peroxidase-linked mouse anti-rabbit antibody (sc-2357, Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at room temperature. Bound antibodies were visualized using a Luminol substrate (Sigma-Aldrich). Chemiluminescence was detected using Image Station 4000MM PRO (Carestream, Toronto, Canada). The membrane was then incubated with horseradish peroxidase-linked mouse monoclonal anti-β-actin antibody (sc-47778 HRP, Santa Cruz Biotechnology) for 1 h at room temperature and chemiluminescence was again detected. Density of bands was measured with the use of ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

Cell growth inhibition assay. The effects of calcitriol and tacalcitol on the cell growth of all cell lines were measured using the MTT assay, which is based on measurements of mitochondrial dehydrogenase activity in living cells (17). Briefly, cells were seeded at a density of 2000 cells per well in 96-well cell culture plates one day prior to the assay and maintained at 37˚C in 5% CO_2. Cells were then treated with calcitriol or tacalcitol alone at four concentrations in the range of 1 to 1000 nM or 5-FU alone at four concentrations in the range of 0.1 to 100 μg/ml (0.768-768.77 μM), or 10 nM calcitriol or tacalcitol together with 5-FU at four concentrations in the range of 0.768-768.77 μM for 120 h. The solvent (ethanol) used at the highest concentration in the assay (1%) did not cause any cytotoxicity. The percentages of inhibition of growth of treated cells were calculated relative to untreated cells (18). The IC_{50} value for 5-FU was defined as the concentration of 5-FU (in the absence or presence of calcitriol or tacalcitol) that is required for half-maximal (50%) inhibition of cell growth compared to the growth of cells not treated with 5 FU. The

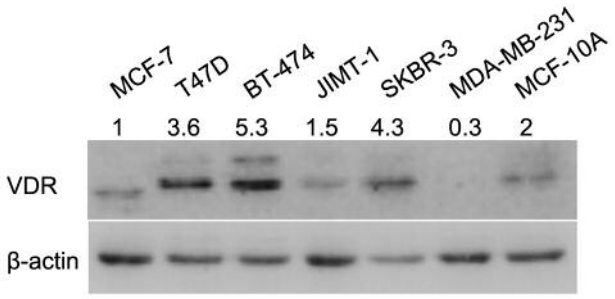

Figure 1. *Expression of the VDR protein in selected human breast cancer and epithelial cell lines. VDR and β-actin proteins were detected in whole cell lysate samples by western blot analysis. Protein levels were quantified by densitometry and expressed above the bands as relative densitometry units of VDR to β-actin ratio and in relation to the protein level in MCF-7 cells. Representative blot is shown.*

IC_{50} values were calculated based on Cheburator 0.4 software (18). In each experiment, samples containing specific concentrations of the preparation were used in triplicate. The experiments were repeated four times.

Statistical analysis. Data are presented as arithmetic means±standard deviations in samples from four independent experiments. The decrease in IC_{50} for a sample of cells treated with 5-FU in the presence of the VDR agonist compared to a sample without the VDR agonist was statistically evaluated by one-tailed *t*-test. The difference between two independent variables was considered statistically significant if the *p*-value was less than 0.05.

Results and Discussion

According to the IARC TP53 Database (p53.iarc.fr), cell lines MCF-7 (luminal A) and MCF-10A (untransformed mammary epithelial) have wild-type *TP53*, while the other cell lines presented in this report have mutated *TP53*. MCF-7 cells have been shown to have low levels of p53, in contrast to T47D (luminal A) and MDA-MB-231 (TNB) cells, which have high levels of p53 encoded by mutated genes (16). Treatment with 10 nM calcitriol or tacalcitol for 120 h, when used alone, inhibited the growth of MCF-7 and SKBR-3 cells only: 17% (calcitriol) and 12% (tacalcitol) inhibition for MCF-7 cells and 23% (calcitriol, tacalcitol) inhibition for SKBR-3 cells, respectively (see footnote to Table I - negative values indicate increased growth). To determine the ability of VDR agonists to potentiate the growth inhibitory effect of 5-FU, cells were incubated with various concentrations of 5-FU for 120 h in the absence or presence of 10 nM calcitriol or tacalcitol. After incubations, MTT assays were performed and 5-FU concentrations causing 50% inhibitory effect (IC_{50}) were calculated. The mean IC_{50} values are shown in Table I. VDR agonists have been found to significantly reduce the IC_{50} of 5-FU after treatment of MCF-7 and BT-474 cells. A downward trend in IC_{50} was also observed in other cell lines, except for MDA-MB-231, where no decrease in the IC_{50} of 5-FU by VDR agonists was observed. This is presumably due to the lack or low level of VDR protein in MDA-MB-231 cells (Figure 1). In turn, T47D cells were shown to have higher levels of VDR proteins than MCF-7 cells (Figure 1), and therefore the reason for the slightly different effects of VDR agonists on their sensitivity to 5-FU is not certain, especially since 10 nM tacalcitol reduced the IC_{50} of DNA-targeting cisplatin to a greater extent in T47D cells than in MCF-7 cells in earlier studies (19). Both MCF-7 and T47D lines represent the luminal A subtype of breast cancer, although they differ in p53 status, which may affect the 5-FU antiproliferative activity in combination with the VDR agonist, which is more cell-cycle specific than the antiproliferative activity of cisplatin in combination with the VDR agonist. Despite some differences in VDR expression, the ratio of IC_{50} for 5-FU alone to the IC_{50} for 5-FU with tacalcitol for all cell lines except MDA-MB-231, was in the range of 1.5-2.2. In conclusion, VDR agonists, calcitriol and tacalcitol, significantly increased sensitivity to 5-FU in at least two luminal subtype breast cancer cell lines among the six examined breast cancer cell lines representing various molecular subtypes.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

Authors' Contributions

Dagmara Klopotowska performed the experiments and analyzed the data. Janusz Matuszyk analyzed the data and wrote the manuscript. All Authors have agreed to the final version of the article.

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References

1 Longley DB, Harkin DP and Johnston PG: 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer *3(5)*: 330-338, 2003. PMID: 12724731. DOI: 10.1038/nrc1074

2 Petrelli F, Ghidini M, Lonati V, Tomasello G, Borgonovo K, Ghilardi M, Cabiddu M and Barni S: The efficacy of lapatinib and capecitabine in HER-2 positive breast cancer with brain metastases: A systematic review and pooled analysis. Eur J Cancer *84*: 141-148, 2017. PMID: 28810186. DOI: 10.1016/j.ejca.2017.07.024

3 Behera RK and Nayak R: Expression profiling of nucleotide metabolism-related genes in human breast cancer cells after treatment with 5-fluorouracil. Cancer Invest *27(5)*: 561-567, 2009. PMID: 19219653. DOI: 10.1080/07357900802620802

4 Welsh J: Function of the vitamin D endocrine system in mammary gland and breast cancer. Mol Cell Endocrinol *453*: 88-95, 2017. PMID: 28579119. DOI: 10.1016/j.mce.2017.04.026

5 Lopes N, Paredes J, Costa JL, Ylstra B and Schmitt F: Vitamin D and the mammary gland: a review on its role in normal development and breast cancer. Breast Cancer Res *14(3)*: 211, 2012. PMID: 22676419. DOI: 10.1186/bcr3178

6 Duffy MJ, Murray A, Synnott NC, O'Donovan N and Crown J: Vitamin D analogues: Potential use in cancer treatment. Crit Rev Oncol Hematol *112*: 190-197, 2017. PMID: 28325259. DOI: 10.1016/j.critrevonc.2017.02.015

7 Wietrzyk J, Pełczyńska M, Madej J, Dzimira S, Kuśnierczyk H, Kutner A, Szelejewski W and Opolski A: Toxicity and antineoplastic effect of (24R)-1,24-dihydroxyvitamin D_3 (PRI-2191). Steroids *69(10)*: 629-635, 2004. PMID: 15465107. DOI: 10.1016/j.steroids.2004.05.015

8 Saramäki A, Banwell CM, Campbell MJ and Carlberg C: Regulation of the human p21(waf1/cip1) gene promoter *via* multiple binding sites for p53 and the vitamin D_3 receptor. Nucleic Acids Res *34(2)*: 543-554, 2006. PMID: 16434701. DOI: 10.1093/nar/gkj460

9 Cheng HT, Chen JY, Huang YC, Chang HC and Hung WC: Functional role of VDR in the activation of p27Kip1 by the VDR/Sp1 complex. J Cell Biochem *98(6)*: 1450-1456, 2006. PMID: 16518840. DOI: 10.1002/jcb.20780

10 Pálmer HG, Sánchez-Carbayo M, Ordóñez-Morán P, Larriba MJ, Cordón-Cardó C and Muñoz A: Genetic signatures of differentiation induced by 1alpha,25-dihydroxyvitamin D_3 in human colon cancer cells. Cancer Res *63(22)*: 7799-7806, 2003. PMID: 14633706.

11 Milczarek M, Rossowska J, Klopotowska D, Stachowicz M, Kutner A and Wietrzyk J: Tacalcitol increases the sensitivity of colorectal cancer cells to 5-fluorouracil by downregulating the thymidylate synthase. J Steroid Biochem Mol Biol *190*: 139-151, 2019. PMID: 30923017. DOI: 10.1016/j.jsbmb.2019.03.017

12 Le François BG, Maroun JA and Birnboim HC: Expression of thymidylate synthase in human cells is an early G(1) event regulated by CDK4 and p16INK4A but not E2F. Br J Cancer *97(9)*: 1242-1250, 2007. PMID: 17923872. DOI: 10.1038/sj.bjc.6604020

13 Milczarek M, Psurski M, Kutner A and Wietrzyk J: Vitamin D analogs enhance the anticancer activity of 5-fluorouracil in an in vivo mouse colon cancer model. BMC Cancer *13*: 294, 2013. PMID: 23777514. DOI: 10.1186/1471-2407-13-294

14 Abu El Maaty MA, Dabiri Y, Almouhanna F, Blagojevic B, Theobald J, Büttner M and Wölfl S: Activation of pro-survival metabolic networks by 1,25(OH)(2)D(3) does not hamper the sensitivity of breast cancer cells to chemotherapeutics. Cancer Metab *6*: 11, 2018. PMID: 30181873. DOI: 10.1186/s40170-018-0183-6

15 Lou YR, Molnár F, Peräkylä M, Qiao S, Kalueff AV, St-Arnaud R, Carlberg C and Tuohimaa P: 25-Hydroxyvitamin D(3) is an agonistic vitamin D receptor ligand. J Steroid Biochem Mol Biol *118(3)*: 162-170, 2010. PMID: 19944755. DOI: 10.1016/j.jsbmb.2009.11.011

16 Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A and Gray JW: A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell *10(6)*: 515-527, 2006. PMID: 17157791. DOI: 10.1016/j.ccr.2006.10.008

17 Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods *65(1-2)*: 55-63, 1983. PMID: 6606682. DOI: 10.1016/0022-1759(83)90303-4

18 Nevozhay D: Cheburator software for automatically calculating drug inhibitory concentrations from in vitro screening assays. PloS One *9(9)*: e106186, 2014. PMID: 25184280. DOI: 10.1371/journal.pone.0106186

19 Pelczynska M, Switalska M, Maciejewska M, Jaroszewicz I, Kutner A and Opolski A: Antiproliferative activity of vitamin D compounds in combination with cytostatics. Anticancer Res *26(4A)*: 2701-2705, 2006. PMID: 16886680.

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