Combined Treatment of Breast Cancer Cell Lines with Vitamin D and COX-2 Inhibitors

MARC THILL[1], KATHRIN REICHERT[2], ALENA WOESTE[2], STEPHAN POLACK[2], DOROTHEA FISCHER[2], FRIEDERIKE HOELLEN[2], ACHIM RODY[2], MICHAEL FRIEDRICH[3] and FRANK KÖSTER[2]

[1]Department of Gynecology and Obstetrics, Agaplesion Markus Hospital, Frankfurt am Main, Germany;
[2]Department of Gynecology and Obstetrics, University of Lübeck, Lübeck, Germany;
[3]Department of Gynecology and Obstetrics, Helios Hospital Krefeld, Krefeld, Germany

Abstract. *Background: Vitamin D is known for its anti-cancerogenous potential. Prostaglandin E_2 (PGE_2) is a proliferation and inflammation activating agent. The production of PGE_2 is dependent on the activity of cyclooxygenase-2 (COX-2). A link between vitamin D and PGE_2 metabolism was recently shown. Materials and Methods: In MDA-MB-231 and MCF-7 breast cancer cell lines we investigated the influence of calcitriol and the COX-2 inhibitor celecoxib regarding cell growth via MTT test, as well as on the protein and mRNA expression of COX-2 using western blot and qRT-PCR. Results: The proliferation of MCF-7 and MDA-MB-231 was inhibited by both calcitriol and the COX-2 inhibitor celecoxib and even stronger by their combination. Moreover, calcitriol inhibited the COX-2 protein expression in MDA-MB-231, as well as the COX-2 mRNA expression in both cell lines. Conclusion: The combination of calcitriol and celecoxib demonstrated a cooperative growth-inhibiting effect in breast cancer cell lines.*

The estimated annual incidence of breast cancer in the USA for 2014 for women is 232,670 with an estimated number of deaths of 40,000 female patients (1). In Germany, 70,340 new cases were diagnosed in 2010 and the estimated number for 2014 is 75,200. In 2010, 17,466 breast cancer patients died in Germany (2). Thus, breast cancer is the most significant malignancy in females. Because of the increasing number of patients, there is an unmet need for new preventive strategies and new treatment approaches.

Correspondence to: Frank Köster, Department of Gynecology and Obstetrics, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany. Tel: +49 451500 2163, Fax: +49 4515004905, e-mail: frank.koester@uksh.de

Many advances in breast cancer treatment have been made in the last decade. Many biological approaches led to a targeted and more effective treatment and gene expression profiling towards a better understanding over breast cancer and its sub-groups. Despite these advances, numerous new promising targets warrant further exploration. Two of these targets are cyclooxygenase-2 (COX-2), the key enzyme required to convert arachidonic acid to prostaglandins (PG), and calcitriol [1,25-dihydroxycholecalciferol or 1,25-$(OH)_2D_3$], which is the biologically active form of vitamin D (3).

Calcitriol is known to suppress cell growth, tumor growth and inhibit metastasis, as well as prolong survival in animal models (4). More specifically, it inhibits the growth of human breast cancer cell lines (5-6). Multiple epidemiological studies have suggested that vitamin D has a protective effect against the carcinogenesis and progression of breast cancer (7-10). However, concerning the correlation between vitamin D intake and breast cancer risk, data are still conflicting (9-13).

Calcitriol has been shown to exhibit significant anti-inflammatory actions in breast cancer cells (14, 15). The pro-inflammatory PGs play an important role in the development and progression of breast cancer (16).

Increasing attention has been paid to the role of inflammatory microenvironment in carcinogenesis. The interaction of inflammatory cytokines, growth factors and oncogene activation has been engaged in the fast induction of cyclooxygenase-2 (COX-2) expression during carcinogenesis as COX-2 affects tumor progression by participating in the malignant proliferation, invasion and metastasis (17). High expression of COX-2 is associated with increased proliferation, invasion, apoptotic resistance and angiogenesis (18, 19) and is, therefore, associated with a poor prognosis for cancer patients (20).

Several epidemiological, pre-clinical and clinical studies support the idea that COX-targeting non-steroidal anti-inflammatory drugs (NSAIDs) are playing protective roles against breast cancer (21-24). Consequently, we hypothesize

that a combination of COX-2 inhibition and calcitriol could exert an increased anti-proliferative effect on breast cancer cells and could offer new prevention or treatment approaches in the future. A link between vitamin D and PGE_2 metabolism (25-27) in breast cancer tissue (28-29) has been already proposed in our recent publications.

The aim of the current study was to evaluate a possible cooperative activity of calcitriol, as the active form of vitamin D, and the COX-2 inhibitor celecoxib in breast cancer cells.

Materials and Methods

Cell culture. MCF-7 and MDA-MB-231 breast cancer cell lines were purchased from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The cells were grown in RPMI1640 (Life-Technologies, Darmstadt, Germany) complemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (both from PAA-Laboratories, Cölbe, Germany), at 37°C in a humidified atmosphere with 5% CO_2.

Growth experiments. To compare the proliferation rate between treated cells we used MTT assays. Briefly, 3,000 cells/well were plated on a 96-well microplate. After 24 h, media were changed to contain 3% FCS and test substances were added, all of which were resolved and diluted in DMSO; 1,000 × stock solutions for all calcitriol concentrations and 10,000 × stock solution for all celecoxib (both from Sigma, Taufkirchen, Germany). All solvent controls contained 0.1% DMSO. Six wells were treated for each concentration of each test substance. After 72 h of treatment, the medium was replaced by the 10% MTT solution (5 mg/ml thiazolyl blue in PBS; Sigma) for a further 4-hour incubation period. Then, the reaction was stopped and dissolution of formazan crystals was performed in the dark overnight by adding 100 μl stop-solution (10% (w/v) SDS, 50% N,N-dimethylformamide, pH 4.7). A microplate-reader (Dynatech Laboratories-MRX, DPC Biermann, Bad Nauheim, Germany) was used to measure the absorption of the wells at 560 nm.

Protein expression. To compare the COX-2 protein expression, 300,000 cells were plated on 6-well plates in growth medium for 24 h. Afterwards, treatment was performed in media without FCS for 24 h. Total protein was extracted by using the MPER-buffer (GE healthcare, München, Germany). Equal amounts of protein were analyzed in western blots with a monoclonal COX-2 antibody (clone CX229; Biomol, Hamburg, Germany and a β-actin antibody (clone AC-15; Sigma). Immunoreactive bands were detected with the Immobilon-Western-Chemiluminescent-HRP-Substrate on Hyperfilm-ECL-Performance-Chemiluminescent-Film (both from GE Healthcare). The films were scanned and a densitometric analysis of the protein bands was performed using the Easy-Win software (Herolab, Wiesloch, Germany).

Expression of COX-2 mRNA. For the relative comparison of *COX-2* mRNA expression, the quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used. Therefore, 300,000 cells per well were plated on a 6-well plate in growth media and the media were replaced after 24 h by fresh media containing 3% FCS together with the test substances. After 24 h of treatment, total RNA was isolated by using QIAzol (Qiagen, Hilden, Germany). For the

reverse transcription, 1μg RNA was used together with random primers and Superscript-II (Invitrogen, Karslruhe, Germany). For the qPCR, the Platinum-SYBR-Green-qPCR-Super-Mix-UDG (Invitrogen) was used. As primers for *COX-2* we used the Hs_PTGS2_1_SG-QuantiTect-Primer-Assay (Qiagen) and for the hypoxanthine-guanine phosphoribosyltranferase 1 (*HPRT1*) we used the forward: 5'-TCA GGC AGT ATA ATC CAA AGA TGG T-3' and reverse: 5'-AGT CTG GCT TAT ATC CAA CAC TTC G-3' primers (synthesized at Metabion, Martinsried, Germany). The PCR protocol consisted of 42 cycles for 15 s at 95°C and 30 s at 60°C. Each sample was tested in duplicates. Data were further processed with the excel-based program REST-MCS©-version 2 (Qiagen).

Results

Treatment of breast cancer cell lines with calcitriol. Calcitriol significantly decreases the growth of MCF-7 breast cancer cells at tested concentrations between 100 nM and 10 μM to 71.5% and 36.8%, respectively, compared to the solvent control (Figure 1A). The growth of MDA-MB-231 was also inhibited by 10 μM calcitriol to 88.4% (Figure 1B). Combined treatment with calcitriol and celecoxib. The growth of breast cancer cell lines was inhibited by 10 μM calcitriol as already seen in the experiment above. Furthermore, growth was significantly inhibited by 10 μM celecoxib in MCF-7 and MDA-MB-231 cells to 67.8 and 88.4%, respectively. In MCF-7, the combination of 10 μM celecoxib and 10 μM calcitriol was significantly stronger than that of calcitriol-alone (Figure 2A, a) or celecoxib-alone (Figure 2A, b). One μM celecoxib significantly decreased the growth of MCF-7 and, furthermore, the addition of 10 μM calcitriol and 1 μM celecoxib inhibited the growth to a greater extent than 1 μM celecoxib alone (Figure 2A, c). In MDA-MB-231 cells, the combinations of 10 μM celecoxib and 1 μM calcitriol (Figure 2B, a), as well as 10 μM calcitriol (Figure 2B, b), inhibited the growth significantly stronger than 10 μM celecoxib-alone. The combination of 10 μM celecoxib and 10 μM calcitriol was stronger than 10 μM calcitriol-alone (Figure 2B, c).

COX-2 protein and mRNA expression under the influence of calcitriol and celecoxib. The COX-2 protein could be detected in western blots of MDA-MB-231 cells (Figure 3) but not of MCF-7. After 6 h of treatment with 0.1 or 1% calcitriol, the density of the western blot signal for COX-2 was decreased in both concentrations to 62.7% compared to the solvent control. Celecoxib had no effect on the COX-2 protein and the combination of celecoxib and calcitriol decreased the COX-2 protein to 87%. The results were quantified using densitometry and normalized with the immunoreactive signals of β-actin. *COX-2* mRNA expression in MDA-MB-231 and MCF-7 cells was decreased after a 3-h treatment with celecoxib to 0.78±0,19 and 0.78±0.32, respectively, as well as with calcitriol to 0.59±0.23 and 0.58±0.32, respectively (Table I). It is remarkable, that nearly
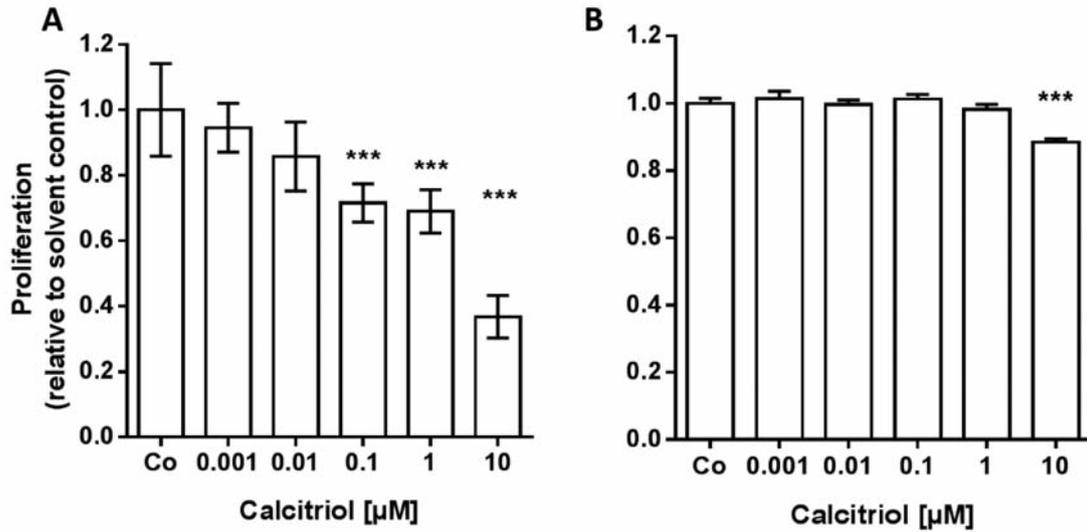
Figure 1. *The influence of calcitriol on the growth of breast cancer cell lines. Cell growth of MCF-7 and MDA-MB-231 cells was assessed by the MTT assay after 72 h of treatment with various concentrations of calcitriol. The results are shown as mean±SEM; statistical significance: ***p<0.001; (Co, control).*
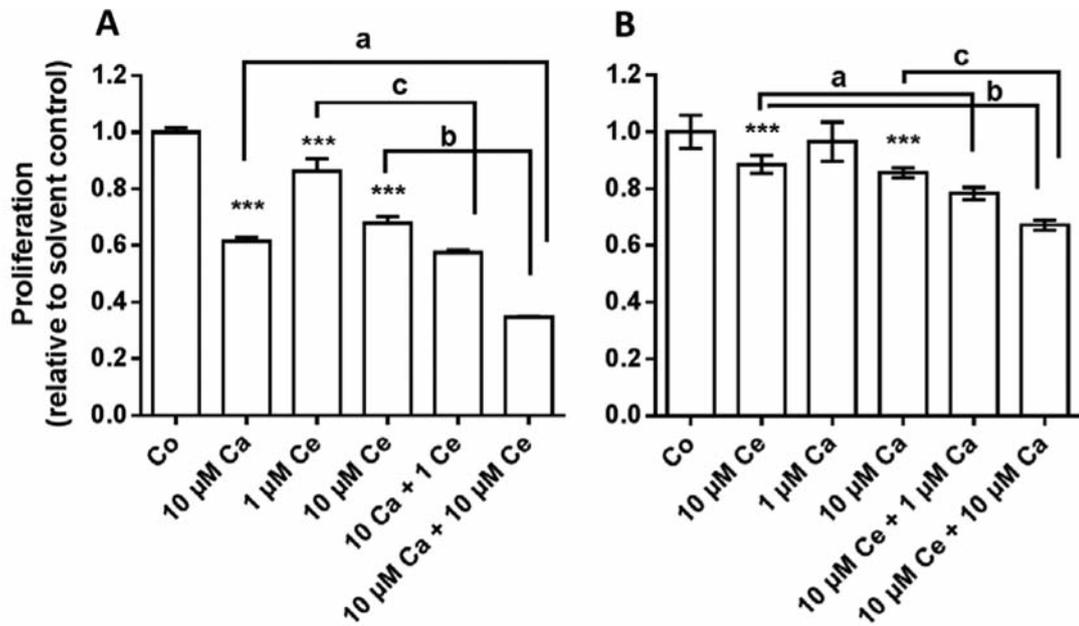

Figure 2. *Combined treatment with calcitriol and celecoxib on MCF-7 (A) and MDA-MB-231 (B) cells. The proliferation is shown as the mean±SEM of cultures compared to the solvent control. Statistical significance: ***p<0.001. For the combined treatments, compared to single treatments, (A): a, b and c=p<0.001 and (B): a=p<0.01, b and c=p<0.001 (Co, control; Ca, calcitriol; and Ce, celecoxib).*

the same decline in mRNA expression was found in both cell lines after treatment with celecoxib and calcitriol, because the content of *COX-2* mRNA in MCF-7 seemed to be extremely low, when compared to MDA-MB-231. The latter statement is based on an estimation deduced from the comparison of PCR-cycle-time differences between the *HPRT-1* and *COX-2* of both cell lines. The combined treatment with celecoxib and calcitriol hardly affected the *COX-2* mRNA expression. The inflammatory IL-1β increased *COX-2* mRNA in the MCF-7 cells to 2.57-fold.
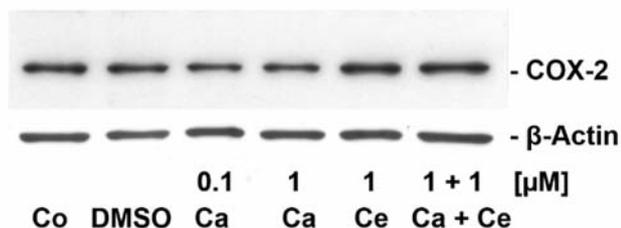
Figure 3. *COX-2 protein expression in MDA-MB-231 cells after treatment with calcitriol and celecoxib. Western blot using COX-2 antibody and β-actin as a loading control (Co, control; Ca, calcitriol; Ce, celecoxib).*

Table I. *Measurement of COX-2 mRNA after treatment with calcitriol and celecoxib. Results are the mean±SEM of treated cells versus the untreated solvent control.*

	Celecoxib	Calcitriol	Celecoxib + Calcitriol	IL-1β
MCF-7	0.78±0.19	0.59±0.23	0.95±0.29	2.57±0.46
MDA-MB-231	0.78±0.32	0.58±0.24	1.17±0.37	1.27±0.99

Discussion

In the present study, we observed an inhibition of the proliferation in two breast cancer cell lines by both calcitriol and the selective COX-2 inhibitor celecoxib. We also recorded an additive inhibition of their combination. This additive inhibition in breast cancer cell lines is shown for the first time.

Colston and Hansen have summarized several studies and clarified that calcitriol can influence the cell cycle, cell differentiation, invasion and apoptosis of breast cancer cells *in vitro* (30). In a recently published study, we showed an inhibition of breast cell proliferation by calcitriol in MDA-MB-231, MCF-7 and MCF-10F cells (27). This is in line with data by Yuan *et al*. (17) who showed that 1,25-(OH)$_2$D$_3$ significantly inhibited the proliferation of MCF-7 cells in a time- and dose-dependent manner using MTT assays. In contrast to the results of Yuan *et al*., we used higher concentrations of calcitriol to receive comparable inhibitory effects what might be explained by different growth conditions.

The main focus of our study was to treat breast cancer cell lines with the combination of calcitriol and the COX-2 inhibitor celecoxib. The growth inhibition of breast cancer cell lines that we observed by celecoxib has been shown previously (31-32); *e.g.* by Dai *et al*. who observed a time- and dose-dependent inhibition of proliferation in both MCF-7 and MDA-MB-231. In contrast to Dai *et al*. we used lower concentrations of celecoxib and only one treatment time of 72 h (32). At 10 μM celecoxib we observed a comparable reduction of proliferation than Dai *et al*.. Furthermore, we combined the application of calcitriol with celecoxib, each at 10 μM and observed significant additive effects compared to each single application. Therefore, we suggest that the combination of both substances might be a valuable tool for the targeted-treatment of breast cancer cells.

A further interesting question was to examine the connection, if any, between vitamin D and prostaglandin pathways. Therefore, we measured COX-2, the central PG-

synthesizing enzyme, after treatment with calcitriol. A decrease of the COX-2 protein content in MDA-MB-231, as well as in the mRNA level in both tested breast cancer cell lines was found. Yuan *et al*. also addressed this issue and was able to show a decrease of COX-2 protein in MCF-7 cells; we were unable to detect the COX-2 protein in MCF-7 (17). Why Yuan *et al*. were able to detect COX-2 in western blot and we were not, might be due to a possibly different version of the cell line or different culture conditions. For us, it did not seem to be unusual that the COX-2 protein content in MCF-7 was under the level of detection, since we were estimating the *COX-2* mRNA level relative to *HPRT-1* to be approximately 1,000-times lower compared to MDA-MB-231. After treatment with celecoxib the COX-2 protein level was not decreased in MDA-MB-231. Moreover, celecoxib seemed to counteract the inhibiting effect of calcitriol. Regarding the *COX-2* mRNA our results are in line with Dai *et al*. showing a decrease in the COX-2 mRNA after treatment with celecoxib, However, in our study the mRNA was analyzed after 6 h and in the study of Dai *et al*. after 48 h (32).

Down-regulation of COX-2 expression by calcitriol is an important issue in breast cancer, because it is limiting the synthesis and biological actions of pro-inflammatory PGs (5).

Epidemiological studies support a protective effect of vitamin D. In a recently published meta-analysis the authors concluded that low blood levels of calcitriol are associated with an increased risk of recurrence and death of breast cancer patients (10). However, regarding the prevention of breast cancer by vitamin D supplementation, data are conflicting. While in the results of the meta-analysis by Sperati *et al*. vitamin D supplementation was not reducing the breast cancer risk (13), Chen *et al*. stated an inverse relationship between vitamin D intake and breast cancer risk (33).

To date, calcitriol has been evaluated in a number of preclinical and some clinical studies as an antitumor agent in different carcinomas; reviewed in Trump *et al*. (34). Up to now, the maximum tolerated dose of calcitriol is still unclear and the application of calcitriol is recommended to be used restrictively due to its potential hypercalcaemic impact (35-36). Only a sparse number of clinical trials regarding the use of calcitriol in combination with chemotherapy for cancer

treatment exist with unsatisfying results (34). The ASCENT-I-Trial (AIPC Study of Calcitriol Enhancing Taxotere) evaluated the combination of docetaxel and calcitriol in pre-treated and advanced prostate cancer patients but the study was stopped because the treatment arm of the study was associated with shorter survival compared to the control arm (37).

The importance of COX inhibitors is widely recognized, since they suppress breast cancer cell growth both *in vivo* and *in vitro* (16, 38). The inhibition of COX-2 by the selective inhibitor celecoxib apparently inactivates the transcription of aromatase and, thus, inhibits the proliferation of tumor cells in oestrogen-responsive breast cancer (39). Furthermore, celecoxib is able to induce apoptosis in different types of cancer (40). Epidemiological studies support the hypothesis of breast cancer prevention by non-steroidal inflammatory drug (NSAID) administration. Several studies, including one meta-analysis, reported a breast cancer risk reduction after NSAIDs use between 16% and 40% (23-24, 41-42). In different murine models for breast cancer celecoxib reduced tumor growth, increased apoptosis and reduced neo-angiogenesis (43-44).

The correlation between COX-2 and estrogen metabolism is an important issue. The inhibition of aromatase expression due to COX-2 inhibition is proposing an interesting therapeutic strategy. Until now, several clinical trials have been conducted to investigate the use of COX-2 inhibitors regarding their impact on breast cancer. The German Breast Group (GBG) has completed a multi-center clinical phase-III study, the REACT trial, which analyzes the combination of endocrine treatment and celecoxib in primary breast cancer. Various other combination therapies with COX-2 inhibitors regarding the metastatic setting were completed with inconsistent data (45-48). In a preoperative decision-making setting, two studies have recently been published (49-50): In the study by Brandao *et al*. patients were randomized to receive either pre-operative 400 mg celecoxib twice daily for two to three weeks. The impact on proliferation was reflected by a reduction of Ki-67-positive cells (49). However, Martin *et al*. found, in a similar setting, no statistical significant changes in Ki-67 expression. (50). The first data suggest a possible treatment approach for a combination of COX-2 inhibitors and calcitriol in breast cancer (25) as both prostaglandin and calcitriol metabolism influence carcinogenesis and tumor growth. The metabolisms of calcitriol and prostaglandin are linked together by various factors and thus a synergistic effect might be supposed. The group of Moreno *et al*. has demonstrated growth inhibition by COX-2 inhibitors and calcitriol in prostate cancer cells. They used benign and malignant prostate cell lines and illustrated that calcitriol inhibits the PG-dependent proliferation of prostate cancer cells. Moreover, the authors showed that a combination of calcitriol with a COX-2 inhibitor had a synergistic effect on the growth inhibition of prostate cancer cells (51).

We conclude that the combination of the COX-2 inhibitor celecoxib and calcitriol are cooperatively inhibiting the growth of breast cancer cell lines. Calcitriol decreases aromatase expression by direct repression, as well as *via* an indirect effect due to the reduction in the levels of PGs (5), and celecoxib supports this suppressive effect on oestrogen synthesis. Therefore, we suggest a therapeutic role of the combination of celecoxib and calcitriol in hormone receptor-positive breast cancer, probably with the addition of an aromatase inhibitor. Based on our findings, we believe that it is worth to consider prospective clinical trials showing the beneficial actions of celecoxib and calcitriol in endocrine-responsive breast cancer.

References

1 Cancer Facts & Figures 2014. (American Cancer Society, Atlanta, 2014).

2 Krebs in Deutschland 2009/2010., Vol. 9 (Robert Koch-Institut (Hrsg) und die Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. (Hrsg). Berlin, 2013).

3 Thill M, Terjung A and Friedrich M: Breast cancer – new aspects of tumor biology: are calcitriol and cyclooxygenase-2 possible targets for breast cancer? Eur J Gynaecol Oncol *35*: 341-358, 2014.

4 Honma Y, Hozumi M, Abe E, Konno K, Fukushima M, Hata S, Nishii Y, DeLuca HF and Suda T: 1 alpha,25-Dihydroxyvitamin D_3 and 1 alpha-hydroxyvitamin D_3 prolong survival time of mice inoculated with myeloid leukemia cells. Proc Natl Acad Sci USA *80*: 201-204, 1983.

5 Krishnan AV, Swami S and Feldman D: The potential therapeutic benefits of vitamin D in the treatment of estrogen receptor positive breast cancer. Steroids *77*: 1107-1112, 2012.

6 Welsh J, Wietzke JA, Zinser GM, Smyczek S, Romu S, Tribble E, Welsh JC, Byrne B and Narvaez CJ: Impact of the Vitamin D3 receptor on growth-regulatory pathways in mammary gland and breast cancer. J Steroid Biochem Mol Biol *83*: 85-92, 2002.

7 Goodwin PJ, Ennis M, Pritchard KI, Koo J and Hood N: Prognostic effects of 25-hydroxyvitamin D levels in early breast cancer. J Clin Oncol *27*: 3757-3763, 2009.

8 Janowsky EC, Lester GE, Weinberg CR, Millikan RC, Schildkraut JM, Garrett PA and Hulka BS: Association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk. Public Health Nutr *2*: 283-291, 1999.

9 Kim Y and Je Y: Vitamin D intake, blood 25(OH)D levels, and breast cancer risk or mortality: a meta-analysis. Br J Cancer *110*: 2772-2784, 2014.

10 Rose AA, Elser C, Ennis M and Goodwin PJ: Blood levels of vitamin D and early stage breast cancer prognosis: a systematic review and meta-analysis. Breast Cancer Res Treat *141*: 331-339, 2013.

11 McCullough ML, Rodriguez C, Diver WR, Feigelson HS, Stevens VL, Thun MJ and Calle EE: Dairy, calcium, and vitamin D intake and postmenopausal breast cancer risk in the Cancer Prevention Study II Nutrition Cohort. Cancer Epidemiol Biomarkers Prev *14*: 2898-2904, 2005.

12 Rossi M, McLaughlin JK, Lagiou P, Bosetti C, Talamini R, Lipworth L, Giacosa A, Montella M, Franceschi S, Negri E and La Vecchia C: Vitamin D intake and breast cancer risk: a case-control study in Italy. Ann Oncol *20*: 374-378, 2009.

13 Sperati F, Vici P, Maugeri-Sacca M, Stranges S, Santesso N, Mariani L, Giordano A, Sergi D, Pizzuti L, Di Lauro L, Montella M, Crispo A, Mottolese M and Barba M: Vitamin D supplementation and breast cancer prevention: a systematic review and meta-analysis of randomized clinical trials. PLoS One 8: e69269, 2013.

14 Krishnan AV and Feldman D: Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. Annu Rev Pharmacol Toxicol 51: 311-336, 2011.

15 Krishnan AV, Trump DL, Johnson CS and Feldman D: The role of vitamin D in cancer prevention and treatment. Endocrinol Metab Clin North Am 39: 401-418, table of contents, 2010.

16 Hoellen F, Kelling K, Dittmer C, Diedrich K, Friedrich M and Thill M: Impact of cyclooxygenase-2 in breast cancer. Anticancer Res 31: 4359-4367, 2011.

17 Yuan L, Jiang R, Yang Y, Ding S and Deng H: 1,25-Dihydroxyvitamin D3 inhibits growth of the breast cancer cell line MCF-7 and downregulates cytochrome P4501B1 through the COX-2/PGE$_2$ pathway. Oncol Rep 28: 2131-2137, 2012.

18 Chang SH, Liu CH, Conway R, Han DK, Nithipatikom K, Trifan OC, Lane TF and Hla T: Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. Proc Natl Acad Sci USA 101: 591-596, 2004.

19 Zha S, Yegnasubramanian V, Nelson WG, Isaacs WB and De Marzo AM: Cyclooxygenases in cancer: progress and perspective. Cancer Lett 215: 1-20, 2004.

20 Ristimaki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H and Isola J: Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res 62: 632-635, 2002.

21 Allott EH, Tse CK, Olshan AF, Carey LA, Moorman PG and Troester MA: Non-steroidal anti-inflammatory drug use, hormone receptor status, and breast cancer-specific mortality in the Carolina Breast Cancer Study. Breast Cancer Res Treat 147: 415-421, 2014.

22 Harris RE, Chlebowski RT, Jackson RD, Frid DJ, Ascenseo JL, Anderson G, Loar A, Rodabough RJ, White E and McTiernan A: Breast cancer and nonsteroidal anti-inflammatory drugs: prospective results from the Women's Health Initiative. Cancer Res 63: 6096-6101, 2003.

23 Harris RE, Namboodiri KK and Farrar WB: Nonsteroidal antiinflammatory drugs and breast cancer. Epidemiology 7: 203-205, 1996.

24 Sharpe CR, Collet JP, McNutt M, Belzile E, Boivin JF and Hanley JA: Nested case-control study of the effects of non-steroidal anti-inflammatory drugs on breast cancer risk and stage. Br J Cancer 83: 112-120, 2000.

25 Thill M, Hoellen F, Becker S, Dittmer C, Fischer D, Kummel S, Salehin D, Friedrich M, Koster F, Diedrich K and Cordes T: Expression of prostaglandin- and vitamin D-metabolising enzymes in benign and malignant breast cells. Anticancer Res 32: 367-372, 2012.

26 Fischer D, Thome M, Becker S, Cordes T, Diedrich K, Friedrich M and Thill M: 25-Hydroxyvitamin D$_3$ 1alpha-hydroxylase splice variants in benign and malignant ovarian cell lines and tissue. Anticancer Res 29: 3627-3633, 2009.

27 Thill M, Cordes T, Hoellen F, Becker S, Dittmer C, Kummel S, Salehin D, Friedrich M, Diedrich K and Koster F: Influence of calcitriol on prostaglandin- and vitamin D-metabolising enzymes in benign and malignant breast cell lines. Anticancer Res 32: 359-365, 2012.

28 Cordes T, Hoellen F, Dittmer C, Salehin D, Kummel S, Friedrich M, Koster F, Becker S, Diedrich K and Thill M: Correlation of prostaglandin metabolizing enzymes and serum PGE$_2$ levels with vitamin D receptor and serum 25(OH)$_2$D$_3$ levels in breast and ovarian cancer. Anticancer Res 32: 351-357, 2012.

29 Thill M, Fischer D, Kelling K, Hoellen F, Dittmer C, Hornemann A, Salehin D, Diedrich K, Friedrich M and Becker S: Expression of vitamin D receptor (VDR), cyclooxygenase-2 (COX-2) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in benign and malignant ovarian tissue and 25-hydroxycholecalciferol (25(OH$_2$)D$_3$) and prostaglandin E2 (PGE$_2$) serum level in ovarian cancer patients. J Steroid Biochem Mol Biol 121: 387-390, 2010.

30 Colston KW and Hansen CM: Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. Endocr Relat Cancer 9: 45-59, 2002.

31 Bocca C, Bozzo F, Bassignana A and Miglietta A: Antiproliferative effects of COX-2 inhibitor celecoxib on human breast cancer cell lines. Mol Cell Biochem 350: 59-70, 2011.

32 Dai ZJ, Ma XB, Kang HF, Gao J, Min WL, Guan HT, Diao Y, Lu WF and Wang XJ: Antitumor activity of the selective cyclooxygenase-2 inhibitor, celecoxib, on breast cancer in Vitro and in Vivo. Cancer Cell Int 12: 53, 2012.

33 Chen P, Hu P, Xie D, Qin Y, Wang F and Wang H: Meta-analysis of vitamin D, calcium and the prevention of breast cancer. Breast Cancer Res Treat 121: 469-477, 2010.

34 Trump DL, Deeb KK and Johnson CS: Vitamin D: considerations in the continued development as an agent for cancer prevention and therapy. Cancer J 16: 1-9, 2010.

35 Aparna R, Subhashini J, Roy KR, Reddy GS, Robinson M, Uskokovic MR, Venkateswara Reddy G and Reddanna P: Selective inhibition of cyclooxygenase-2 (COX-2) by 1alpha,25-dihydroxy-16-ene-23-yne-vitamin D$_3$, a less calcemic vitamin D analog. J Cell Biochem 104: 1832-1842, 2008.

36 Masuda S and Jones G: Promise of vitamin D analogues in the treatment of hyperproliferative conditions. Mol Cancer Ther 5: 797-808, 2006.

37 Scher HI, Jia X, Chi K, de Wit R, Berry WR, Albers P, Henick B, Waterhouse D, Ruether DJ, Rosen PJ, Meluch AA, Nordquist LT, Venner PM, Heidenreich A, Chu L and Heller G: Randomized, open-label phase III trial of docetaxel plus high-dose calcitriol versus docetaxel plus prednisone for patients with castration-resistant prostate cancer. J Clin Oncol 29: 2191-2198, 2011.

38 Rozic JG, Chakraborty C and Lala PK: Cyclooxygenase inhibitors retard murine mammary tumor progression by reducing tumor cell migration, invasiveness and angiogenesis. Int J Cancer 93: 497-506, 2001.

39 Diaz-Cruz ES, Shapiro CL and Brueggemeier RW: Cyclo-oxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. J Clin Endocrinol Metab 90: 2563-2570, 2005.

40 Jendrossek V: Targeting apoptosis pathways by Celecoxib in cancer. Cancer Lett 332: 313-324, 2013.

41 Ashok V, Dash C, Rohan TE, Sprafka JM and Terry PD: Selective cyclooxygenase-2 (COX-2) inhibitors and breast cancer risk. Breast 20: 66-70, 2011.

42 Khuder SA and Mutgi AB: Breast cancer and NSAID use: a meta-analysis. Br J Cancer 84: 1188-1192, 2001.

43 Barnes NL, Warnberg F, Farnie G, White D, Jiang W, Anderson E and Bundred NJ: Cyclooxygenase-2 inhibition: effects on tumour growth, cell cycling and lymphangiogenesis in a xenograft model of breast cancer. Br J Cancer *96*: 575-582, 2007.

44 Yoshinaka R, Shibata MA, Morimoto J, Tanigawa N and Otsuki Y: COX-2 inhibitor celecoxib suppresses tumor growth and lung metastasis of a murine mammary cancer. Anticancer Res *26*: 4245-4254, 2006.

45 Canney PA, Machin MA and Curto J: A feasibility study of the efficacy and tolerability of the combination of Exemestane with the COX-2 inhibitor Celecoxib in post-menopausal patients with advanced breast cancer. Eur J Cancer *42*: 2751-2756, 2006.

46 Chow LW, Yip AY, Loo WT, Lam CK and Toi M: Celecoxib anti-aromatase neoadjuvant (CAAN) trial for locally advanced breast cancer. J Steroid Biochem Mol Biol *111*: 13-17, 2008.

47 Dirix LY, Ignacio J, Nag S, Bapsy P, Gomez H, Raghunadharao D, Paridaens R, Jones S, Falcon S, Carpentieri M, Abbattista A and Lobelle JP: Treatment of advanced hormone-sensitive breast cancer in postmenopausal women with exemestane alone or in combination with celecoxib. J Clin Oncol *26*: 1253-1259, 2008.

48 Falandry C, Debled M, Bachelot T, Delozier T, Cretin J, Romestaing P, Mille D, You B, Mauriac L, Pujade-Lauraine E and Freyer G: Celecoxib and exemestane *versus* placebo and exemestane in postmenopausal metastatic breast cancer patients: a double-blind phase III GINECO study. Breast Cancer Res Treat *116*: 501-508, 2009.

49 Brandao RD, Veeck J, Van de Vijver KK, Lindsey P, de Vries B, van Elssen CH, Blok MJ, Keymeulen K, Ayoubi T, Smeets HJ, Tjan-Heijnen VC and Hupperets PS: A randomised controlled phase II trial of pre-operative celecoxib treatment reveals anti-tumour transcriptional response in primary breast cancer. Breast Cancer Res *15*: R29, 2013.

50 Martin LA, Davies GL, Weigel MT, Betambeau N, Hills MJ, Salter J, Walsh G, A'Hern R and Dowsett M: Pre-surgical study of the biological effects of the selective cyclo-oxygenase-2 inhibitor celecoxib in patients with primary breast cancer. Breast Cancer Res Treat *123*: 829-836, 2010.

51 Moreno J, Krishnan AV, Swami S, Nonn L, Peehl DM and Feldman D: Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. Cancer Res *65*: 7917-7925, 2005.