Induction of apoptosis and inhibition of prostate and breast cancer growth by BGP-15, a new calcipotriene-derived vitamin D_3 analog

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The role of vitamin D_3 in cancer prevention and its potential as an anticancer therapeutic agent have been researched and are well established. However, the clinical use of the natural vitamin D_3 metabolite, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$ or calcitriol] is limited by a possible cause of hypercalcemia and hypercalciuria. A new 24-chloro calcipotriene-based vitamin D_3 analog (BGP-15) was synthesized and examined for antiproliferative activity in the androgen-dependent cell lines of prostate cancer (LNCaP) and breast cancer (MCF-7). The new analog led to significant decrease in cell viability in cultured LNCaP and MCF-7 cell lines compared with calcipotriene and 1,25$(OH)_2D_3$. We observed elevated vitamin D receptor protein levels in both LNCaP and MCF-7 cells, which were treated with 5 μmol/l of 1,25$(OH)_2D_3$, calcipotriene or BGP-15 for 20 h, indicating vitamin D receptor-binding ability. Treatments of LNCaP and MCF-7 cells with 5 μmol/l BGP-15 and calcipotriene for 20 h generated procaspase-3 cleavage and therefore, apoptosis. Interestingly, BGP-15, and to a lesser extent calcipotriene, but not 1,25$(OH)_2D_3$, activated caspase-3 in MCF-7 cells, a cell line that normally lacks this specific caspase (and procaspase). It is presumed that management of MCF-7 with BGP-15 modulates procaspase-3 expression and cleavage, and a subsequent activation of caspase-3. Similar treatments of LNCaP cells induced procaspase-9 cleavage and therefore caspase-9 activation, whereas similar treatments of MCF-7 cells failed to induce caspase-9 activation. Cytochrome c release was, however, detected in both cell lines, LNCaP and MCF-7. In-vivo results suggested that BGP-15 (similar to its parent drug) did not cause calcium-related toxic side effects after chronic treatment. *Anti-Cancer Drugs* **21:609–618 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2010, **21**:609–618

Keywords: apoptosis, calcipotrioene, caspase, vitamin D_3, vitamin D receptor

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Received 13 November 2009 **Revised form accepted** 25 January 2010

Introduction

1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$, also known as calcitriol] is the biologically active metabolite of vitamin D_3, a member of the steroid hormone super family. Vitamin D_3 is known mainly for its major metabolic function in the physiological regulation of mineral transport, mineral metabolism, and bone homeostasis [1,2]. Therefore, it is often used in the treatment of metabolic rickets, osteomalacia, hypoparathyroidism, and osteoporosis [3]. During the last decade, it has become clear that vitamin D_3 participates in a variety of biomechanisms within normal and cancer human cells, including the regulation of differentiation, proliferation, and apoptosis. The role of vitamin D_3 in cancer prevention and its potential as an anticancer therapeutic agent have been researched and are well established in a variety of cancer cells *in vitro* and in tumors *in vivo* [4,5]. Moreover, altered expressions of different proteins, such as, enzymes, which are crucial in vitamin D synthesis and catabolism, have been observed in many tumor types, that is, reduction in the activity of 25-hydroxyvitamin D_3-1α hydroxylase [6]. Treatments of different kinds of human cancer cell-lines and tumors with 1,25$(OH)_2D_3$ have shown to induce arrest in the cell cycle, mainly in the G_1 phase [5,7]. In several cases, including prostate carcinoma, the cell-cycle arrest is associated with increased expression of the proteins $p21^{Waf1}$ and $p27^{Kip1}$, both cyclin-dependent kinases inhibitors [4,8,9]. In addition to its antiproliferative effect, 1,25$(OH)_2D_3$ was shown to suppress the growth effect of two vitamin D receptor (VDR)-expressing prostate cancer cell-lines by inducing apoptosis through release of cytochrome c from the mitochondria followed by the activation of caspase-9 and caspase-3 [9]. Treatment with 1,25$(OH)_2D_3$ has also shown to inhibit invasiveness of human breast cancer cells *in vitro* [10], and several tumors *in vivo* [11,12]. Hence, 1α,25$(OH)_2$ vitamin D_3 has been proposed as an anticancer agent and as a novel therapeutic strategy for many human malignancies such as prostate, colon, and breast carcinomas [13]. However, the clinical use of 1,25$(OH)_2D_3$ in anticancer treatments is limited by its toxicity, that is, a possible cause of hypercalcemia and hypercalciuria in treated patients [5,14]. This obvious disadvantage of 1,25$(OH)_2D_3$ has led to an extensive search for efficient vitamin D_3 analogs, which would display at least similar effects on cancer cells but lack its

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328337f3e9

toxic side effects [15–18]. The genomic response of vitamin D_3 and its various analogs is mediated by binding to the VDR, an intracellular ligand-activated transcription factor [2,5]. The VDR receptor is present in more than 30 human tissues including colon and the mammary, and prostate glands [19]. Interestingly, VDR protein levels increase after exposure to vitamin D_3 and its analogs *in vitro* and *in vivo*, apparently as a result of an increase in the transcription of the VDR gene [20] and an increased receptor-protein lifetime after binding [21,22]. It has recently been shown that larger tumors grow in VDR knockout mice, and these mice are relatively resistant to vitamin D_3 growth inhibition [23]. These findings support the long-standing perception that increased levels of VDR protein correlates with increased antitumor efficacy [13].

Calcipotriene (or calcipotriol, CPT) is a well-known vitamin D_3 analog. CPT is considered to be a highly effective topical agent available and is indicated for hyperproliferative skin diseases, such as psoriasis [24–26]. Despite its significant effects and wide availability, its potential as an anticancer therapeutic agent has not been fully investigated and has remained elusive. A great advantage of CPT is that it is 100–200 times less toxic than calcitriol in terms of systemic effect on calcium homeostasis, thus avoiding hypercalciuria, hypercalcemia, and bone calcium mobilization [27]. Our group has synthesized a new vitamin D_3 analog [(1S, 3R, 5Z, 7E, 22E, 24S)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-24-chloro-1,3-diol, or BGP-15], which is based on CPT and is aimed at retaining the reduced calcemic toxicity of the parent drug, while increasing its anti-proliferative potency.

In this study, we analyzed the new vitamin D_3, CPT-based analog, in which the 24-OH has been substituted in the side chain of CPT. As far as we know, there have been no reported examples of analogs of CPT, in which a hydroxyl group at C-24 has been replaced by a chlorine atom. LNCaP and MCF-7 cell-lines were examined in this study, as they are widely used models for two of the most prevalent nonskin malignancies of human prostate and mammary glands, respectively [28–30]. The results indicated that BGP-15 was more potent than CPT and $1,25(OH)_2D_3$, in its ability to inhibit the clonal cell growth of prostate and breast cancer cells *in vitro*. Similar to the treatment with CPT, no systemic effect on calcium (Ca^{2+}) homeostasis by BGP-15 was observed. In addition to the cell growth inhibition studies, we investigated the biochemical mechanism of BGP-15 that may explain in part its cytotoxic effects on cancer cells.

Materials and methods

Synthesis and purification of BGP-15

CPT (50 mg) dissolved in 10 ml ethanol was mixed in a 10 ml HCl solution (1N) and stirred for 4 h at room temperature. The reaction mixture was evaporated under reduced pressure. The reaction's crude mixture was stored, in dark, under nitrogen and at $-20^{\circ}C$. The purification process of the crude BGP-15 was performed by silica gel flash chromatography (medium-pressure liquid chromatography, eluted with 0.5% methanol in $CHCl_3$, 16 ml/min), and the final yield was 77.6%. Purity (greater than 93% in all cases) was measured by high-pressure liquid chromatography (15 : 85 methanol–water, 1 ml/min) and structure elucidation was carried out using nuclear magnetic resonance (NMR), mass spectrometry (ESI-MS, GC-MS, and MALDI) and infrared analysis (FTIR).

^1H NMR spectra were recorded on a Bruker DMX-500 operating at 500.1 MHz, and chemical shifts are reported in parts per million. 1H-NMR (CD_3OD): 6.34–6.32 (d, 1H), 6.10–6.08 (d, 1H), 6.08–6.074 (s, 1H), 5.97–5.92 (dd, 1H) 5.58–5.46 (m, 2H), 5.29 (s, 1H), 4.90 (s, 1H), 4.36 (bs, 1H), 4.13 (bs, 1H), 3.55 (t, 2H), 2.54–2.48 (m, 2H). TLC (5% MeOH in $CHCl_3$): Rf _ 0.45. LC-ESI-MS (positive mode): *m/z*_453.39: 455.40 (ratio of 3 : 1) $[M + Na]^+$; 431.46: 433.44 (ratio of 3 : 1) $[M + H]^+$; 448.3: 450.2 (ratio of 3 : 1) $[M + NH_4]^+$. MALDI-TOF-MS: 453.253: 455.251 (ratio of 3 : 1) $[M + Na]^+$. GC-MS (after silylation with BSTFA): *m/z* 574 (M^+); 484; 443; 394; 339; 249; 217; 197.

Cell lines, cell culture reagents, and laboratory animals

The cell lines were provided by Dr Daniel Fishman (Department of Morphology, Ben-Gurion University, Beer Sheva, Israel). LNCaP cells were grown in the RPMI medium and MCF-7 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% bovine serum and 200 µmol/l L-glutamine, 10 units/ml penicillin and 10 µg/ml streptomycin (Biological Industries, Beth Haemek, Israel). The cells were kept at 37°C in a 5% CO_2 humidified atmosphere. Female Sprague–Dawley Rats (215–225 g) were obtained from the animal facilities at Ben-Gurion University. The animal protocol was reviewed and approved by the institution's Animal Care and Use Committee, which complies with the Israeli Law of Human Care and Use of Laboratory Animals.

Cell viability assay

The direct effect of BGP-15 and CPT, compared with calcitriol, on the viability of cultured cells was assessed *in vitro* using the LNCaP and MCF-7 cells. The cells ($2.5–5 \times 10^3$ per well) were plated in 96-well plates, and allowed to attach for 24 h at 37°C in a 5% CO_2 humidified atmosphere. A fresh cell medium was then prepared with 1 or 5 µmol/l test compounds by adding them in small aliquots of concentrated ethanolic solutions. The concentration of ethanol did not exceed 0.5%. The medium in the plates was replaced by freshly prepared medium. Medium containing 0.5% ethanol vehicle without test

compounds was used as a control. Cell growth at predetermined times was assayed by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Sigma-Aldrich, Rehovot, Israel), and was evaluated by measuring the absorbance at 540 nm after 3 h of incubation in a medium containing 0.5 mg/ml MTT (Sunrise absorbance plate reader, TECAN). All experiments were conducted at least twice, and weighted results from all experiments were averaged.

Immunoblotting analysis

LNCaP and MCF-7 cells were plated in 6 or 15 mm^2 dishes, and allowed to attach for 48 h at 37°C in a 5% CO_2 humidified atmosphere. The medium was then replaced with a fresh medium containing 5 μmol/l test substances and no more than 0.5% ethanol for 20 h. Whole-cell protein samples for immunoblotting were prepared in a buffer (10 mmol/l Tris–HCl, pH 8.0, 1%vol/wt sodium dodecyl sulfate) preheated to 100°C, in which the cells were homogenized by repeated passage through a syringe equipped with 27G needle. Cytosol protein samples were prepared and homogenized in the same way, but in a cold buffer (10 mmol/l HEPES, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl). Samples were then collected into Eppendorf tubes, incubated for 10 min at 100°C, and centrifuged at 14 000 rpm for 10 min. Supernatants were transferred into clean tubes whereas pellets were discarded. Determination of the protein concentrations was conducted using the Bradford assay. Protein from each sample (100 μg) was subjected to electrophoresis in a 10% SDS–PAGE and transferred to pure nitrocellulose blotting membranes (Pall Corporation, Port Washington, New York, USA), by semidry blotting. Membranes were blocked for 1 h at room temperature with Tris (hydroxymethyl) amino methane saline (TBS) containing 0.05% Tween 20 and 5% bovine serum albumin. The membranes were then incubated overnight at 4°C in TBS with the following antibodies (1 : 200 dilution): rat monoclonal anti-VDR, aa 89–105 of human VDR, gamma isoform (Chemicon; Biotest Ltd., Israel), rabbit polyclonal anti caspase-3 (Ab serotec; Enco Scientific Services Ltd., Israel), rabbit polyclonal anti caspase-9 (Calbiochem, Mercury Ltd., Israel) or mouse monoclonal anti cytochrome c (BD Technologies, Durham, North Carolina, USA). Membranes were washed in TBS containing 5% bovine serum albumin and 0.05% Tween 20, and incubated with either goat, antirabbit, rat or mouse (1 : 5000) secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). The intensities of the bands were quantitated using ImageQuant 5.2 image analyzer (Molecular Dynamics Inc., Sunnyvale, California, USA).

Apoptosis and cell cycle analysis

The population of LNCaP cells undergoing apoptosis and its cellcycle stage was evaluated by flow-cytometry. The cells (0.5×10^6) were treated with the test compounds for 1–3 days. Then the cells were collected, washed with cold PBS, fixated in 70% ethanol, and kept at −20°C for 24–48 h. The pellets were washed in cold PBS and suspended in a PBS solution, containing 0.1% Triton-X and 30 mg/ml DNAse-free RNAse A, (Sigma) for 6 h, at room temperature. Approximately 1 min before the cells were analyzed, propidium iodide (Sigma) in PBS was added to make up a final concentration of 10 μg/ml, and the samples were then analyzed by flow cytometry, using a FACSCallibur instrument (BD Bioscience, San Jose, California, USA). Data were processed with FlowJo software version 7.5 (Tree Star, Inc., Ashland, Oregon, USA).

Urinary and plasma calcium measurements

Twelve normal female rats were acclimatized to the laboratory environment and were individually housed in separate metabolic cages. The rats received rat chow (containing 0.8–1.20% calcium) and tap water *ad libitum*. The animals were divided into four groups, each treated daily with intraperitoneal injections of 20 ng of $1,25(OH)_2D_3$, CPT or BGP-15 in a vehicle for 6 days. All three compounds were dissolved in propylene glycol, which was used as a vehicle. Rats in the control group received the vehicle only. Plasma samples and total urine volumes were collected from each rat daily, and analyzed for calcium levels. Calcium levels in rat urine and plasma were evaluated using a Calcium-Arsenazo reagent set, and processed as recommended by the manufacturer (Pointe Scientific, Inc., Canton, Michigan, USA).

Data analysis

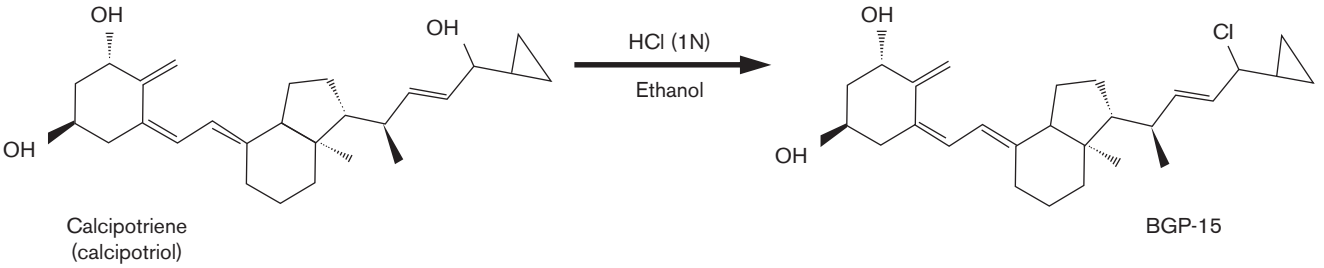
The statistical significance of the difference between the groups was determined by a two-tailed Student's *t*-test. The observations were deemed significant (indicated by asterisks in the figures or tables) if the probability of accepting null hypothesis was less than 0.05. The band densities of the western blots were normalized relative to the relevant control's band density to quantify the differences between the groups. The α-actin bands were not quantified.

Results

Structure of BGP-15

Synthesis and molecular structure of the new analog are outlined in Fig. 1. We have shown that the hydroxyl group at C-24 on the side chain of CPT's structure can simply be replaced by chlorine during a reaction with 1N hydrochloric acid. The natural 25-OH group of calcitriol (and apparently the 24-OH group of CPT) has been widely thought to be necessary for high biological activity, because a fluorine substitution of the side-chain hydroxyl had failed to enhance the calcitriol potency [31]. Although lacking a classical side chain hydroxyl group, BGP-15 was found to be at least as potent as its parent drug or the hormone $1,25(OH)_2D_3$, but less calciuric than the natural hormone.

Fig. 1



Synthesis and molecular structure of (1S,3R,5Z,7E,22E,24S)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-24-chloro-1,3-diol (BGP-15). Calcipotriene dissolved in ethanol was mixed and stirred with 1N HCl solution. The purification process to the crude BGP-15 was performed by silica gel flash chromatography.

The effect of calcipotriene and BGP-15 on the viability of prostate and breast carcinoma cells

The effect of CPT and BGP-15 on the viability of both LNCaP and MCF-7 cells was examined *in vitro* using the MTT assay, and was compared with the effect obtained by the most biologically active metabolite of vitamin D_3-$1,25(OH)_2D_3$. Applications of CPT, BGP-15, and calcitriol at concentrations of 1 and 5 μmol/l resulted in up to 40 and 60% cell death, respectively, in both cultures of LNCaP and MCF-7 cells, after 6 days (Fig. 2). BGP-15 at a concentration of 5 μmol/l was able to reduce LNCaP cell viability at a higher rate than 5 μmol/l CPT and 5 μmol/l $1,25(OH)_2D_3$, that is, 9.56 versus 8.15% and 7.11% reduction per day, respectively. However, the reduction of LNCaP cell viability by BGP-15 treatment was found to be statistically different from 5 μmol/l CPT treatment only, after 5 days. Treatments of MCF-7 cells with 1 or 5 μmol/l of the substances had the same effect as in LNCaP cells on cell viability, generating up to 40 and 60% cell death, respectively, after 6 days. Treatments with $1,25(OH)_2D_3$ (5 μmol/l), CPT, and BGP-15 were slightly more effective in MCF-7 than in LNCaP cells, but no advantage of BGP-15 over the other substances was noted (Fig. 2). On the contrary, a significant reduction was seen after 6 days of cell exposure to 5 μmol/l, but not after 1 μmol/l of BGP-15, when compared with the effect of $1,25(OH)_2D_3$ and CPT. In both LNCaP and MCF-7 cells, the cytotoxic effects of treatments, with a dosage as high as 30 μmol/l of calcitriol, CPT or BGP-15 resembled after 24 h the effects obtained after 6-day treatments with 5 μmol/l of these compounds. The high-dose treatments generated up to 60% cell death in LNCaP cells and up to 50% cell death in MCF-7 cells, after 1 day (data not shown). In summary, all these experiments have shown that BGP-15 is a potent inhibitor of the clonal growth of LNCaP and MCF-7 cells. Furthermore, exposure of LNCaP cells to this new agent at 5 μmol/l concentration exerts a significant antiproliferative effect in comparison with CPT, and especially in comparison with $1,25(OH)_2D_3$.

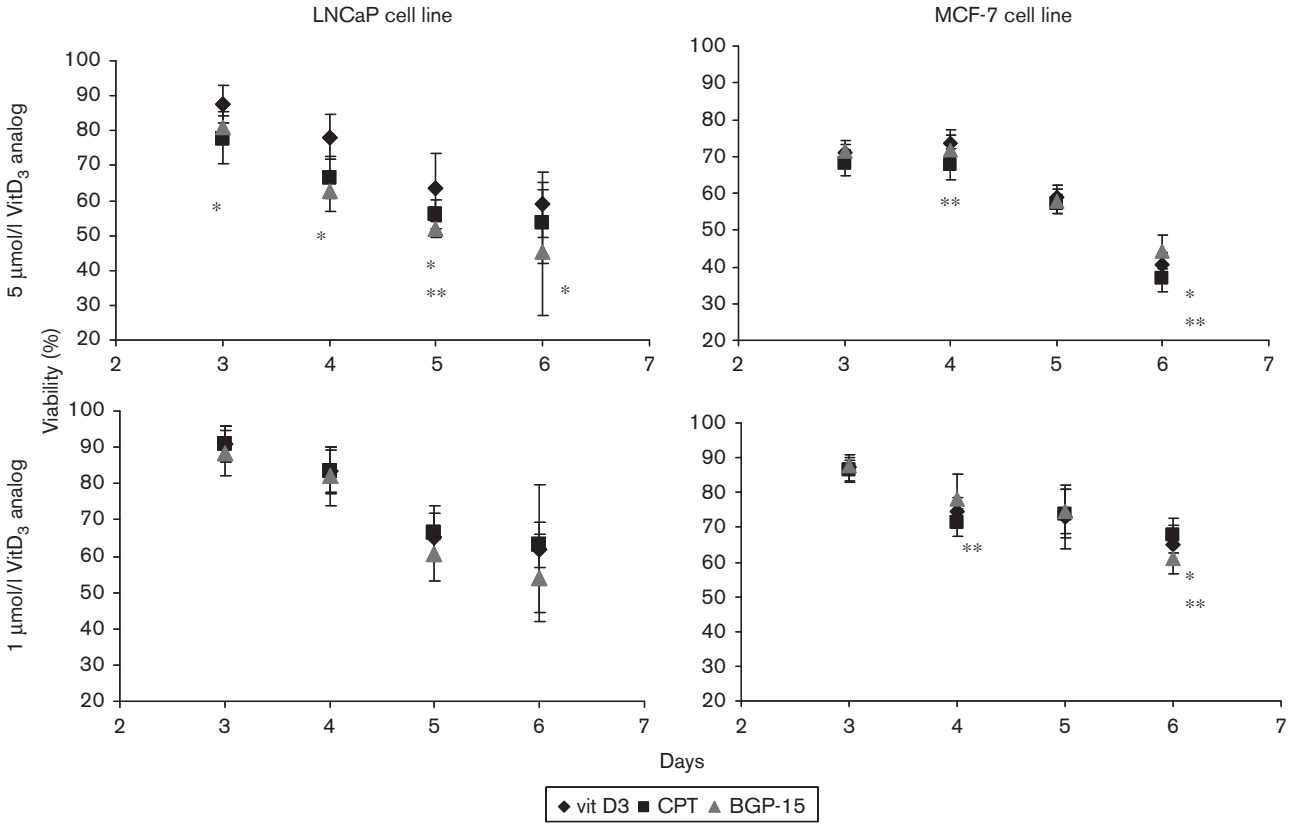
Treatments with BGP-15 and calcipotriene elevate VDR protein levels in LNCaP and MCF-7 cells

As mentioned above, VDR protein levels increase after exposure to $1,25(OH)_2D_3$ and its analogs [21,22]. To investigate the effect of BGP-15 and CPT on endogenous VDR induction, the protein levels of VDR were examined in the LNCaP and MCF-7 cell lines after exposure to these agents. We observed elevated VDR protein levels in both LNCaP and MCF-7 cells, which had been treated with 5 μmol/l of $1,25(OH)_2D_3$, CPT or BGP-15 for 20 h (Fig. 3). It is obvious, therefore, that VDR-binding ability is also attributed to CPT and BGP-15. Moreover, since increased VDR levels correlate with increased antitumor efficacy [23], the elevation in VDR protein levels in both carcinoma cell types, because of CPT, and BGP-15, can be interpreted as an implication of reduction in the surviving ability of cancer cells.

BGP-15 and calcipotriene generate apoptosis through caspase-3 activation and induce cell growth arrest

Exposure of LNCaP and MCF-7 cells to 5 μmol/l CPT and BGP-15 for 20 h generated procaspase-3 cleavage, and therefore, caspase-3 activation. In LNCaP, the caspase-3 activation, which was observed after treatments with BGP-15 and CPT, resembled the activation observed after treatment with $1,25(OH)_2D_3$ (Fig. 4a). An interesting observation was that treatments of MCF-7 cells with CPT and BGP-15 induced procaspase-3 production and cleavage, although MCF-7 breast carcinoma cells do not normally express this specific protease. Treatment of MCF-7 with BGP-15 yielded highly increased caspase-3 protein levels (cleavage) than the levels induced by CPT. As shown in Fig. 4a, the proportions of caspase-3 production in MCF-7 cells after treatment with BGP-15, CPT, and $1,25(OH)_2D_3$ were 25 : 15 : 5, respectively. It was clearly shown that although procaspase-3 is not initially expressed in these cells, CPT, and especially BGP-15, induce expression of the procaspase-3 protein in the cells, followed by cleavage, and therefore, caspase-3 activation. It is interesting to note that unlike CPT and

Fig. 2

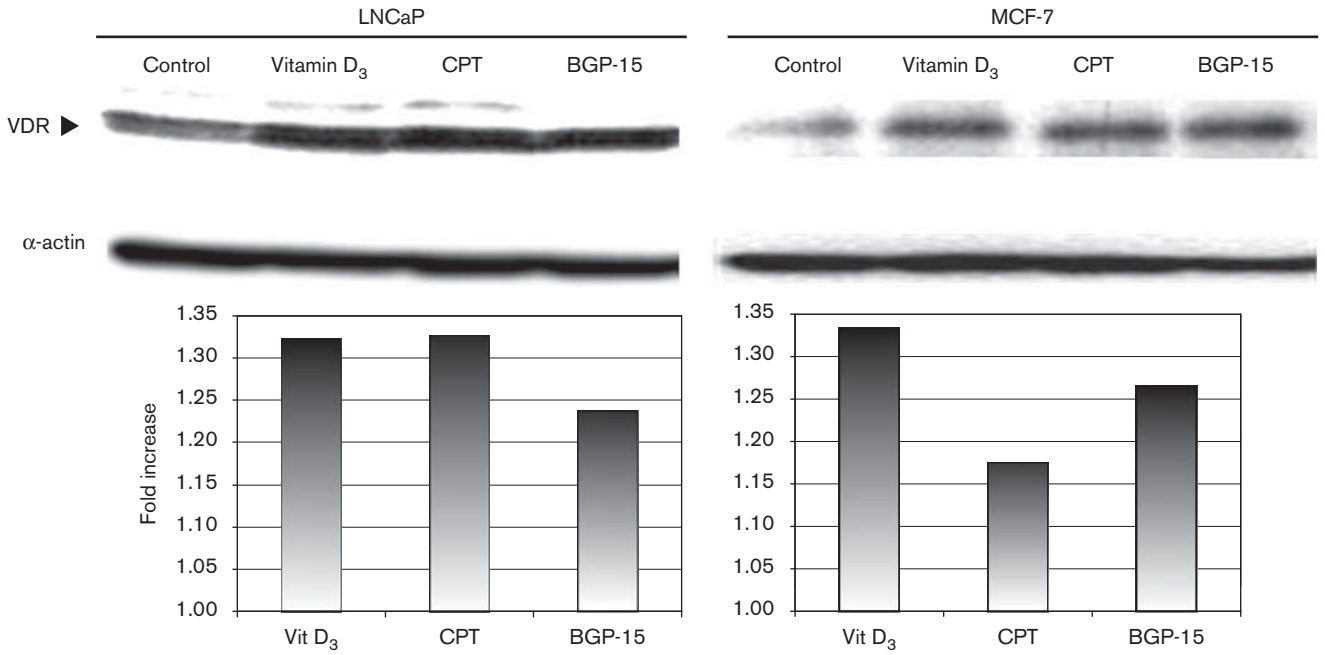


1α, 25-dihydroxyvitamin D_3, calcipotriene and BGP-15 reduce prostate and breast cancer cells' viability. LNCaP and MCF-7 cells were plated in 96-well plates and treated with 1 or 5 µmol/l test substances for 6 days – $1,25(OH)_2D_3$ (vit D_3, ◆), calcipotriene (CPT, ■) and BGP-15 (▲). Medium containing 0.5% ethanol without test compounds was used as a control. Cell viability after treatments was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and the data obtained from the treatment groups were weighted with the vehicle control values, that is, 100% viability in the control group. The weighted results of the decreased cell viability after treatment with the three test compounds are illustrated. Data are expressed as average value ± standard deviation. *$P<0.05$ of BGP-15 relative to vitamin D3, **$P<0.05$ of BGP-15 relative to CPT ($n=16$, Student's *t*-test, two-tailed distribution).

BGP-15, $1,25(OH)_2D_3$ did not actually induce caspase-3 in MCF-7 cells, although the viability of MCF-7 cells after exposure to $1,25(OH)_2D_3$ (5 µmol/l, 6 days) was comparable with the viability obtained after treatment with CPT or BGP-15. These results indicate that (i) BGP-15 and CPT inhibit the growth of cancer cells by a noncytostatic mechanism, and (ii) The mechanism of growth inhibition of MCF-7 by calcipotrience and BGP-15 is different from the mechanistic action of $1,25(OH)_2D_3$ in these cells, but may be similar in the LNCaP cell line. It was also concluded that CPT and its new derivative induce apoptosis through caspase-3 activation in LNCaP and MCF-7 cells. Similar treatments of LNCaP cells induce procaspase-9 cleavage, and therefore, caspase-9 activation (Fig. 4b), whereas similar treatments of MCF-7 cells have failed to induce caspase-9 activation. It should be noted that cytochrome c release was however detected in both the cell lines, LNCaP and MCF-7. Figure 4 presents the release of cytochrome c in MCF-7 cells, which qualitatively shows that this activity was initiated by $1,25(OH)_2D_3$ and CPT, and to a lesser extent, by BGP-15. The above findings indicate that despite the similarities between the two cancer cells, $1,25(OH)_2D_3$ and its analogs may act by different pathways in each cell line.

For the evaluation of LNCaP cell population undergoing late apoptosis, and its cell-cycle condition, cells were exposed to 5 µmol/l $1,25(OH)_2D_3$, CPT or BGP-15 for 1, 2, and 3 days. Cells were collected and analyzed by flow cytometry after propidium iodide staining. It was found that treatment of the cells with $1,25(OH)_2D_3$, CPT or BGP-15 resulted in an incline in the G_1-phase of the cell population, and simultaneously resulted in a significant decrease in the S-phase from the first through the third day, as compared with the vehicle control group of each day (Table 1). After 3 days, all treatments started to generate progressive apoptosis, as observed by the very low DNA content in the cells as a result of DNA

Fig. 3



Vitamin D receptor (VDR) protein levels in whole-cell lysates of LNCaP and MCF-7 cells treated with 5 μmol/l $1,25(OH)_2D_3$ (vit D_3), calcipotriene (CPT) or BGP-15 for 20 h. The band densities were normalized relative to the relevant control's band density to quantify the differences between groups. Bars show the increase in band intensities relative to control.

fragmentation. As shown in Table 1, 9.16% of LNCaP cells were in an apoptotic stage after 3 days after BGP-15 treatment, compared with 0.95%, 1.37%, and 2.69% of cells after vehicle (control), CPT, and $1,25(OH)_2D_3$ treatments, respectively. The analysis showed a prominent cell-cycle arrest of LNCaP cells after their exposure to BGP-15, CPT, and $1,25(OH)_2D_3$. Although all the three treatments were found to be significantly more potent than the vehicle control treatment, in terms of inducing apoptosis, their effects did not significantly differ from one another. As shown in Fig. 2, by the third day of treatments with 5 μmol/l of $1,25(OH)_2D_3$, CPT and BGP-15, LNCaP cells displayed approximately 80% viability relatively to the vehicle control treatment, indicating the death of approximately 20% of the cell population. The gap between this result and the lower extent of apoptosis found after similar treatments can be simply explained by the fact that during the flow cytometry analysis, the dead and necrotic cell population were excluded, and only the cell population, which underwent apoptosis at that point of time was determined. Obviously, this apoptotic fraction was found to be smaller than the total fraction of dying and dead cells.

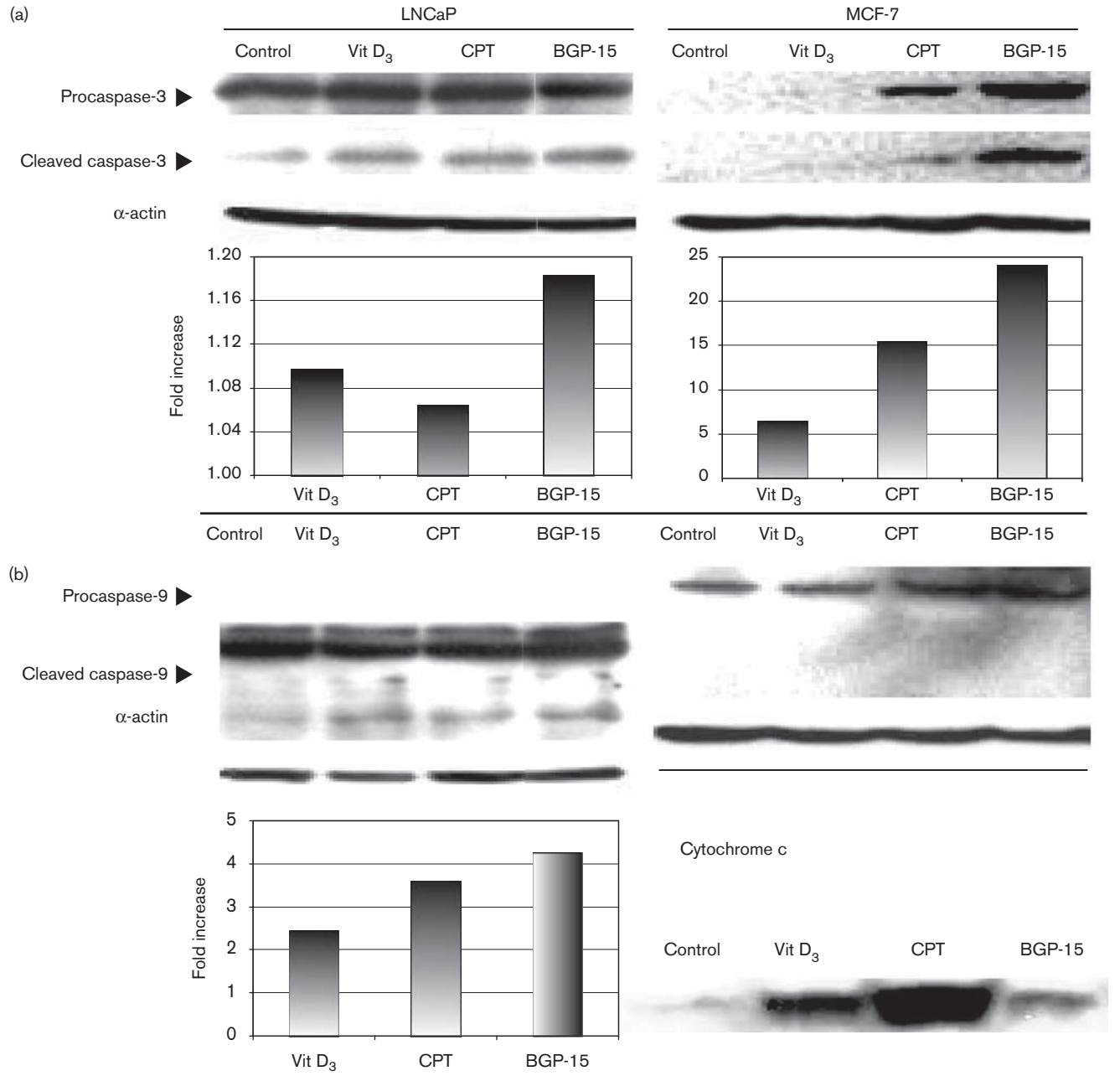
Daily treatments with calcipotriene or BGP-15 does not generate hypercalcemia and hypercalciuria in rats

We evaluated the plasma calcium levels in rats and the total daily secretion of calcium in the rat urine collected, after daily treatments of 20 ng of $1,25(OH)_2D_3$, CPT or BGP-15, for 6 days. Our tests showed no change in plasma calcium levels after treatments with either compound (data not shown). As expected, we observed that treatments with $1,25(OH)_2D_3$ led to a 50% increase in the daily amounts of urinary calcium, as shown after the fourth day of the treatments (Table 2). However, the rats that received CPT or BGP-15 had normal Ca^{2+} levels during the treatment period. Both the treatments with CPT and BGP-15 had no hypercalciuric effect, which means that BGP-15, like its parent drug, is probably not able to cause toxic side effects during treatments. After 3 weeks of the study and 6 days of treatments with 20 ng/day of the tested compounds, all animals had survived.

Discussion

The vast majority of publications on vitamin D_3 analogs deal with the structure–function relationship for understanding and assessing biological profiles of the analogs. It was concluded in various studies that the 1α-hydroxyl group is more important for calcium mobilization, antiproliferative and gene transcriptional activities than the presence of natural 25-OH on the side chain of the hormone [32–34]. Furthermore, it was clearly shown that structural changes on the A-ring, such as 1α-F,25(OH)-16-ene-23-yne-D_3 [32], 1α-CHF_2,25(OH)D_3 or 1α-CH_2OH,25(OH)D_3 [33,34], 1α,25$(OH)_2$-5,6-*trans*-D_3 [35], 2-methylene-19-norvitamin D_3 analog [36], 4,4-difluoro-1α,25$(OH)_2D_3$, or 19-fluoro-1α,25$(OH)_2D_3$ [37], resulted in less biologically potent molecules than

Fig. 4



(a) Caspase-3 and (b) caspase-9 activation (procaspase cleavage) in LNCaP and MCF-7 cells following treatments with 5 μmol/l 1,25(OH)2D3 (vit D_3), calcipotriene (CPT) or BGP-15 for 20 h. The band densities were normalized relative to the relevant control's band density to quantify the differences between groups. Bars show the increase in cleaved procaspase as quantified band intensities relative to control.

the natural $1\alpha,25(OH)_2D_3$. However, modifications in D-ring (i.e. 16-ene) and/or in the side chain, such as $1\alpha,25(OH)_2$-16-ene,23-yne-D_3 [32,35], 1α(OH)-16-ene-25-SO_2-CF_3-D_3, and 1α(OH)-20-epi-22-oxa-26-SO_2-CF_3-D_3 [38] or *tert*-butyl ketone analogs (24 and 25-oxo) [39,40] significantly increased the antiproliferative activity. Posner *et al.* [33] showed that substitution of 25-OH by 25-CHF_2 did not make a difference in the antiproliferative activity, but substitution of 1α-OH with CHF_2 generated a less antiproliferative analog. This is, therefore, an indication that the side-chain hydroxyl is not as important or necessary for antiproliferative activity. Although the necessity of the side-chain hydroxyl is in question, it is discussed by Grzywacz *et al.* [36], who have shown that the total removal of the 25-OH from 2-methylene-19-nor-$1\alpha,25(OH)_2D_3$ reduces the in-vitro potency. Numerous studies of $1\alpha,25(OH)_2D_3$ analogs with a 'potentiating' side chain have been published, in which fluorine atoms

Table 1 Population of LNCaP cells undergoing apoptosis and cell cycle stages

	1-day treatment			2-day treatments			3-day treatments		
	Apoptosis	G_1	S	Apoptosis	G_1	S	Apoptosis	G_1	S
Vehicle	0.84 (±0.36)	71.07 (±1.19)	13.38 (±1.78)	0.26 (±0.21)	68.51 (±4.75)	15.98 (±3.75)	0.95 (±1.22)	70.15 (±1.22)	14.13 (±0.63)
$1,25(OH)_2D_3$	0.54 (±0.65)	78.47 (±3.40)	8.63 (±1.52)[a]	0.42 (±0.40)	82.72 (±2.95)	7.92 (±1.99)[a]	2.69 (±0.28)[a]	81.20 (±0.11)	7.97 (±1.12)[a]
CPT	0.56 (±0.71)	80.52 (±1.38)	8.28 (±2.31)[a]	0.66 (±0.41)	76.62 (±2.61)	10.27 (±2.37)[a]	1.37 (±0.74)[a]	75.91 (±2.68)	10.31 (±0.62)[a]
BGP-15	0.39 (±0.40)	78.86 (±1.82)	7.87 (±0.41)[a]	0.46 (±0.24)	83.32 (±2.07)	7.44 (±1.71)[a]	9.16 (±6.71)[a]	75.79 (±2.63)	6.86 (±1.99)[a]

The evaluation was following treatments with 5 µmol/l of $1,25(OH)_2$ vitamin D_3 (calcitriol), calcipotriene (CPT) or BGP-15 for 1, 2, and 3 days. Data are expressed as average ± standard deviation. Values in the brackets are the standard deviations.
[a]$P < 0.05$ relative to the control (vehicle) experimented on the same day.

Table 2 Urinary calcium measured in rats (mg/day) following daily injections of $1,25(OH)_2D_3$, calcipotrience, or BGP-15

Day	1	2	3	4	5	6
Vehicle	2.5 (±0.1)	2.0 (±0.4)	0.8 (±0.5)	1.3 (±0.3)	1.1 (±0.3)	1.2 (±0.4)
$1,25(OH)_2D_3$	2.1 (±0.9)	2.4 (±0.9)	1.3 (±1.0)	3.2 (±1.3)	2.9 (±0.9)	3.3 (±0.8)
Calcipotriene	2.5 (±0.8)	1.7 (±0.5)	1.3 (±0.4)	1.3 (±0.2)	1.0 (±0.3)	1.4 (±0.3)
BGP-15	2.2 (±0.8)	1.5 (±0.5)	1.5 (±0.6)	1.2 (±0.1)	1.3 (±0.6)	1.4 (±0.4)

Data are expressed as average ± standard deviation. Values in the brackets are the standard deviations. [Normal female rats were individually housed in separated metabolic cages. They were then divided into four groups (3 rats per group), each was treated daily with intraperitoneal injections of 20 ng of the stated substances for 6 days. Animals in the control group received propylene glycol (vehicle) alone. Calcium levels in urine were evaluated using the Calcium-Arsenazo reagent set].

were introduced to the side chain, either containing or lacking the 25-OH substituent [33,34,38,41,42]. It has been hypothesized that by incorporating fluorine atoms to the side chain there would be a hydrogen-bonding surrogate for the natural 25-hydroxyl group of calcitriol [34]. We have adopted a similar strategy by substituting the 24-OH in the CPT sidechain with a chlorine atom. By only modifying the hydroxyl group on the side chain, we have clearly shown that this hydroxyl group is really not important for the analog's activity. Moreover, we have shown for the first time that replacement of this hydroxyl group by chlorine (and probably by other halogens) enables potentiating the antiproliferative activity.

We examined the effects of the new 24-chloro-dehydroxy-calcipotriene analog, or BGP-15, on cancer cells using two-cultured human cell lines-LNCaP prostate carcinoma and MCF-7 breast carcinoma. Our results have shown that BGP-15 is as potently antiproliferative as CPT. As shown, 6-day treatments with 5 µmol/l CPT or BGP-15 resulted in up to 60% mortality in LNCaP and MCF-7 cells. Treatments of MCF-7 with $1,25(OH)_2D_3$, CPT and BGP-15 produced similar effects on the cell viability throughout the 6 days of treatments with 5 µmol/l compounds, whereas, similar treatment of LNCaP cells with BGP-15 reduces the viability of those cells more potently than both $1,25(OH)_2D_3$ and CPT. It may indicate that BGP-15 would be more active in prostate carcinoma treatments than $1,25(OH)_2D_3$ and CPT, or at least would have a similar potency. It has been shown earlier in carcinomas of the prostate and mammary glands that $1,25(OH)_2D_3$ induced arrest in the cellcycle, mainly in the G_1 phase [5,7]. Our data have shown that like the treatment of LNCaP cells with $1,25(OH)_2D_3$, treatments with CPT or BGP-15 increased the number of cells in the G_1-phase of the cell-cycle and significantly decreased the number of cells in the S-phase simultaneously. The significant decrease of about 50% in the S-phase suggests that treatments of the cells with CPT and its BGP-15 analog, block prostate cancer cells from proceeding from the G_1-phase cell cycle to the next S phase, and thus inhibit proliferation in the treated cells. In addition to the growth inhibition, the treatments of LNCaP cells with $1,25(OH)_2D_3$, CPT or BGP-15 induced apoptosis, which is characterized by chromosomal DNA fragmentation. It is therefore evident that the new BGP-15 and CPT analogs are able to arrest the growth of prostate cancer cells and induce apoptosis.

Caspase-3 is a member of the caspase family of cysteine proteases, and its activation is considered to be responsible for the actual demolition of the cell during apoptosis [43]. As shown, treatment with CPT and BGP-15 generated procaspase-3 cleavage, and therefore caspase-3 activation, in both LNCaP and MCF-7 cells. Caspase-3 activation was induced after treatments of LNCaP cells with each of the three compounds. However, in MCF-7 cells, procaspase-3 cleavage occurred by CPT and BGP-15 but not after $1,25(OH)_2D_3$ treatment. Thus, BGP-15 and CPT activate caspase-3 while processing programmed cell death in both LNCaP and MCF-7 cells, whereas $1,25(OH)_2D_3$ activates this protease in LNCaP cells only. The results regarding MCF-7 cells are new and interesting, since these cells neither express procaspase-3 nor activate caspase-3 [44,45]. Caspase-3 activation in LNCaP cells seems to be a result of a downstream cleavage by caspase-9 [46], which was also activated in these cells after treatments with $1,25(OH)_2D_3$, CPT or BGP-15. This was not the case in MCF-7 cells, where no caspase-9 was observed after treatments with either compound, although the cells expressed endogenous procaspase-9, and cytochrome c was released from the

mitochondria into the cytosol. Mitochondrial cytochrome c release has been known to represent an intrinsic pathway of apoptosis but it can also be induced by the activation of cell surface receptors Fas and TNF followed by activation of caspase-8 that can activate downstream caspases (such as caspase-3, caspase-6, and caspase-7) [47]. Thus, the extrinsic pathway can also cause cytochrome c release, which triggers caspase activation through Apaf1. $1,25(OH)_2D_3$ and the tested analogs, however led to cytochrome c release, but the following step of caspase-9 activation through Apaf1 can be inhibited or blocked in MCF-7 cells. In conclusion, these new data imply that (i) BGP-15 and CPT (and calcitriol) act at least through the mitochondrial 'intrinsic' pathway for apoptosis in LNCaP cells by caspase-9/caspase-3 activation, while the 'extrinsic' pathway involving death receptors cannot be ruled out; and (ii) BGP-15 and at lesser extent, CPT, (but not calcitriol) may act through the 'extrinsic' pathway in MCF-7 cells.

As known, VDR has a significant role in cancer development and survival, while increased levels of VDR protein correlates with increased antitumor efficacy [13,23]. Our results showed that CPT and BGP-15 [such as $1,25(OH)_2D_3$] mediate elevation in VDR protein levels in LNCaP and MCF-7 cells. This indicates that CPT and BGP-15 inhibit cancer cell growth and carry out cytotoxic effects through the intracellular VDR, and treatments of cancer cells with these two agents are likely to result in a weakened cell potency and survival. The role of VDR in the antiproliferative activity of CPT and BGP-15 needs to be further investigated by using, for example, siRNA VDR transfected cells.

Development of vitamin D_3 analogs that exert antiproliferative effects, without producing hypercalcemia, is critical for the successful application of this type of therapy. As known, the clinical use of vitamin D_3 in anticancer treatments is limited by its toxicity [14] and by concerns of a possible development of hypercalcemia or hypercalciuria in treated patients [5]. Therefore, we examined the pharmacological effect of daily treatments with BGP-15 on the calcium levels in the plasma and urine of rats, and compared the data with those obtained after treatments with CPT and $1,25(OH)_2D_3$. The results showed that both treatments with CPT or BGP-15 had no effect on calcium levels in rat urine, whereas treatment with $1,25(OH)_2D_3$ resulted, as expected, in increased amounts of calcium secreted in the urine from the fourth day of the treatment. In addition, our study showed no changes in plasma calcium levels after treatments with $1,25(OH)_2D_3$, CPT or BGP-15. These results suggest that BGP-15, like its parent drug, (at dosage regimen of 20 ng/day) does not cause calcium-related toxic side effects during treatment.

In summary, we have introduced in this paper a new CPT-based analog, BGP-15, in which the hydroxyl group at C-24 was replaced by a chlorine atom. Both BGP-15 and CPT are able to inhibit the growth of two types of cancer cells by induction of apoptosis through mitochondrial cytochrome c release and caspase-3 activation. The apparent discrepancy of cytochrome c release, which is not followed by caspase-9 activation in MCF-7 cells, is an interesting phenomenon that should be further investigated. Although BGP-15 arrests cell growth at a similar rate as $1,25(OH)_2D_3$ and CPT, this agent exhibits higher potency in activating caspase-3 and inducing apoptosis, than the two other compounds. We further showed that BGP-15 (as CPT) does not cause hypercalcemia and hypercalciuria during the treatment. As growth inhibition and apoptosis are important targets in cancer research [48,49], the results presented in this paper justify BGP-15 as a suitable candidate for clinical therapy of prostate and breast cancer. However, further research should be done to elucidate the role of side-chain chlorine (or other halogens) in the biological effects of this vitamin D_3 analog. In addition, in-vivo testing, using an animal model in the setting of low tumor burden is necessary for efficacy evaluation.

Acknowledgements

This study was supported in part by Yeshaya Horowitz Applied Reseach Fund in Biotechnology, Pharmaceutics, and Medical Devices. Parts of this manuscript were presented at the 20th conference of the European Association for Cancer Research (EACR-20), Lyon, France, July 5–8, 2008, and at the Charles Rodolphe Brupbacher Symposium 'Targets for Cancer Prevention and Therapy', Zurich, Switzerland, February 11–13, 2009. The authors are grateful to Daniel Fishman, Shlomit Fedida-Metula, and Yuliana Levin-Gromiko for discussion and technical assistance.

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