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Effect of a Vitamin D₃ derivative (B3CD) with postulated anti-cancer activity in an ovarian cancer animal model

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Summary

The objective of the present study was to test the hypothesis that Calcidiol derivative B3CD qualifies as a potential anti-cancer drug *in vivo* employing an ovarian cancer xenograft model in mice. In addition, the selectivity of B3CD on viability and proliferation of platinum-resistant human ovarian cancer cell lines in comparison to control cell lines was analyzed *in vitro*. B3CD displayed cell line-

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specific cytotoxicity screened against a panel of ovarian and other carcinoma cell lines, endothelial and control cells. B3CD, at sub-cytotoxic concentrations, revealed stronger effects on the proliferation of SKOV-3 ovarian cancer cells vs. primary fibroblasts as determined by BrdU incorporation analysis. Treatment with B3CD at 0.5 μ M resulted in highly condensed chromatin and fragmented nuclei in SKOV-3 cells but not in primary fibroblasts. B3CD induced cell death at low drug concentrations (≤ 0.5 μ M) in SKOV-3 ovarian cancer cells is mediated by the p38 MAPK signaling pathway: B3CD induced p38 MAPK expression and activation in SKOV-3 cells and inhibition of p38 signaling counteracted B3CD induced cell death *in vitro*. An ovarian cancer cell animal model (human SKOV-3 cell derived xenografts in nude mice) revealed that tumor growth in few B3CD treated mice accelerated while the majority of B3CD treated mice displayed delayed tumor growth or full tumor regression. B3CD possesses anti-ovarian cancer properties *in vitro* and *in vivo*. We propose the further development of non-calcemic bromoacetoxy derivatives of vitamin D₃ as potential anti-cancer therapeutics.

Keywords

Calcidiol-derivative B3CD; Ovarian cancer; MAPK-signaling; SKOV-3 xenograft model

Introduction

In 2008, ovarian cancer was the leading cause of death (15,520 cases) from gynecologic malignancies and ranks second (21,650 cases) among newly diagnosed gynecological cancers in the United States [1,2]. Although most patients (70–80%) initially respond to cytoreductive surgery and adjuvant paclitaxel and platinum-based chemotherapy the majority will experience disease recurrence [3–5]. While re-treatment with a platinum-based drug is possible for some women the response rate to current second line or third line (after interim non-platinum therapy) chemotherapy is <33% due to the rise of resistance to these drugs [6–8]. The development of new drugs to treat platinum-resistant tumors alone or in combination with platinum therapy is desired. Calcitriol/vitamin D₃ (1,25-dihydroxy-vitamin D₃), an endocrine hormone responsible for calcium and mineral homeostasis, inhibits cell proliferation and induces differentiation, via binding to the vitamin D receptor (VDR) [9]. Its clinical use in cancer therapy has been proposed but is limited by its hypercalcemic side effects [10–12]. Synthetic analogs of calcitriol/vitamin D₃ have been developed to overcome these side effects but secondary to hypercalcemia their therapeutic efficacy remains limited [13]. The natural precursor to calcitriol/vitamin D₃ is calcidiol. Calcidiol, found abundantly in serum, is a substrate to 1 α -vitamin D hydroxylase, and is biologically inactive in terms of binding to VDR [14]. Because the production of calcitriol/vitamin D₃ by renal 1 α -vitamin D hydroxylase is under tight transcriptional control, an elevation in serum calcidiol level does not necessarily translate into higher serum calcitriol/vitamin D₃ or calcium levels [15] and does not lead to lethal calcemic side effects, even at supra-physiological doses [16]. Past studies led to the development of bromoacetoxy-calcidiol (B3CD), a 3-bromoacetoxy-ester derivative of calcidiol, which was proposed to be a potential new drug to treat cancer [17]. Bromoacetoxy analogs generally display an improved pharmacologic profile, exert less toxicity, and display a greater stability compared to their parent compounds [18,19]. B3CD in recent *in vitro* studies exerted potent anti-proliferative effects on prostate cancer cell lines [17,20,21] and neuroblastoma cell lines [22]. The objective of the present study was to test the recent hypothesis that B3CD qualifies as a potential anti-cancer drug *in vivo* employing an ovarian cancer xenograft model in mice. In addition, the selectivity of B3CD on viability and proliferation of platinum-resistant human ovarian cancer cell lines in comparison to control cell lines was analyzed *in vitro*.

Materials and methods

Synthesis of B3CD

A procedure described earlier, with suitable modifications was used to synthesize Calcidiol derivative B3CD (Fig. 1a) [23,24]. Briefly, equimolar amounts of calcidiol and bromoacetic acid were stirred with excess of dicyclohexylcarbodiimide and dry pyridine in dichloromethane in an ice bath for 4 h. Our modifications entail preparative high performance liquid chromatography (HPLC; Waters, Milford, MA, USA) using a C18 Luna column (4.6×150 mm; 5 µm; Phenomenex (Torrance, CA, USA) of B3CD followed by ¹H NMR and Mass spectroscopy characterization [22].

Cell culture

Human cell lines SKOV-3, OVCAR-3 (ovarian adenocarcinomas), PC-3, LNCaP (prostate carcinoma), BxPC-3 (pancreatic adenocarcinoma), BJ (primary human fore skin fibroblasts) were obtained from American Type Culture Collection (Manassas, VA), HUVEC (human umbilical vein endothelial cells) were obtained from Lonza Inc. (Allendale, NJ) and HTR-8/SVneo (first-trimester cytotrophoblasts with extended lifespan) were provided by Dr. Sharma (Providence, RI, USA). Cells were grown in T75 cell culture flasks (Corning, New York, NY) in complete medium (Gibco, Rockville, MD) according to the suppliers recommendation. For all assays cells after seeding were allowed to attach overnight and were treated in complete medium.

Cell viability assay

Viability of cell lines before and after drug treatment was determined by the CellTiter 96[®] Aqueous One Solution Assay (Promega Corp, Madison, WI) following the manufacturer's recommendations. In this colorimetric assay the resulting OD at 490 nm is directly proportional to the number of living cells [25]. Briefly, cells (5×10³/well) were plated into 96 well flat bottom plates (Corning, Inc., Corning, NY) before treatment with B3CD or vehicle (ethanol) as indicated. For assays with inhibitors (p38 MAPK: #559389/ SB203580, p38 MAPK: #559388/ SB202190, p38 MAPK control: #559387; Erk1/2: #328007, Erk1/2 control: #328008; JNK1/2/3: #420119/ SP600125, JNK1/2/3 control: #420123, Jun/JNK: #420130, Jun/JNK control: #420131; MEK: #513000/PD98059; PI3 kinase: #440202/LY294002; all from Calbiochem, La Jolla, CA) the cells were pre-incubated with 0–40 µM inhibitor for 2 h prior to B3CD (0–3 µM) addition. Following incubation at 37°C in a cell culture incubator for 20 h MTS reagent was added at a 1:10 dilution to the medium. The samples were incubated for an additional 4 h for BxPC-3 or 2 h for all other cell lines before absorbance was measured at 490 nm in an ELISA plate reader (Thermo Labsystems, Waltham, MA). Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations (X±SD) of a representative experiment in percent of absorbance by samples with untreated cells [=100%].

Morphological studies

Cells were seeded (1×10⁴/chamber) into a Lab-Tek[®] Chamber-Slide System (Nalge Nunc., Naperville, IL) and treated for 24 h with 0.5 µM B3CD or calcitriol/vitamin D₃ as a control alongside with non-treated cells. Following two wash-steps in PBS the cells were fixed in PBS, 2% para-formaldehyde (PFA), 0.2% Triton X for 20 min at RT and stained for 10 min with 200 ng/mL 4'-6-Diamidino-2-Phenylindole (DAPI) in 1× phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) before mounting. Representative images were taken with an inverted microscope (Nikon Eclipse TE2000-E, CCD camera) and 20× objective.

Cell proliferation assay

Proliferation of various cell lines was determined by a BrdU (5-bromo-2'-deoxyuridine) incorporation assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations. Briefly, cells (5×10^3 /well) were plated into 96 well flat bottom plates (Corning, Inc., Corning, NY) before treatment as indicated (Results section) for 18 h in FCS free media. BrdU (10 μ M final concentration) was added to the cells and they were further grown for 6 h. After washing, cells were fixed and incubated for 2 h at 37°C with an anti-BrdU antibody-peroxidase conjugate. Immunocomplexes were detected by addition of a tetramethylbenzidine (TMB) substrate solution according to the manufacturer's recommendations. The reaction was stopped by adding 50 μ L of a 1 M sulfuric acid, and the absorbance was measured with an ELISA plate reader (Thermo Labsystems, Waltham, MA) at 450 nm. In this assay, the color intensity correlates directly to the amount of BrdU incorporated into the DNA, which in turn represents proliferation. Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations ($X \pm SD$) of a representative experiment in percent of absorbance by samples with untreated cells [=100%].

Western blot analysis

Cells were seeded into 100 mm² tissue culture dishes (5×10^5 cells/dish), and treated with 1 μ M B3CD for 5 min, 1 h, or 6 h. Preparation of cell lysates, PAGE and immunoblotting was carried out as described previously [26]. Primary antibodies against p38 (#9212), phosphor-p38 (#9215S) and β -actin (#1501) were purchased from Cell Signaling Technology (Beverly, MA) and were diluted 1:1000 in 5% BSA/PBST (phosphate buffered saline with 0.1% Tween 20). As a size standard pre-stained Precision Plus Protein Kaleidoscope (BioRad, Hercules, CA) marker was used. The bands were visualized using horseradish peroxidase-conjugated secondary antibodies (Amersham-Pharmacia Biotech, Piscataway, NJ), followed by enhanced chemiluminescence (Upstate, Waltham, MA) and documented autoradiography (F-B $\times$ 810 Film, Phenix, Hayward, CA).

Ovarian cancer xenograft model

Animals—*In vivo* experiments were carried out at the animal facility of Rhode Island Hospital (RIH), Providence, with strict adherence to the guidelines of the Animal Welfare Committee of RIH and Women & Infants Hospital. Four to six week-old immunodeficient nude mice (NU/NU; strain code 088/homozygous) (Charles River Laboratories, Wilmington, MA) were maintained at a temperature of $22 \pm 1^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$, with a 12 h light/dark cycle.

Treatment—SKOV-3 cells were cultured to 80% confluence, washed in PBS twice, harvested by trypsination, pooled in complete medium, washed in PBS twice, and 2×10^6 cells/inoculate were suspended in 0.1 mL of matrigel and inoculated subcutaneously in the flank of mice. Mice with developing tumors (of max. 10 mm diameter) were randomly assigned to experimental groups. B3CD was prepared as a stock solution of 1 mM in 100% EtOH and diluted 1:40 in PBS for administration. Mice were treated intraperitoneally every other day with either vehicle control (control group; 9 animals) or 300 μ L (0.15 mg/kg body weight) of B3CD (treatment group; 16 animals) for 56 days (8 weeks).

Evaluation—Mice were weighed and tumor size calculated using a caliper every 4–6 days. Mice were euthanized if tumors grew to be greater than 20 mm in diameter, if severe ulcers developed, or if a loss of 15% body weight occurred.

Data analysis

Mean and standard deviation (SD) were calculated. Mean differences were determined by Student's t-test or determined by one-way ANOVA, using the Newman-Keuls test to account for multiple comparisons in post hoc analyses. For animal studies, Kaplan-Meier analysis of tumor growth beyond 15 mm was carried out by treatment group, and a log rank test was applied to test if curves were significantly different. Software used for these analyses was STATA 9.0 (StataCorp, College Station, TX).

Results

Comparative analysis of the cytotoxic effect of B3CD and calcitriol/vitamin D₃ on various human cell lines

In an initial approach to analyze the cytotoxic effects of B3CD (Fig. 1a) on ovarian cancer cells we performed a viability assay using SKOV-3 and OVCAR-3 along with adenocarcinoma cell lines from different tissues (PC-3, prostate; BxPC-3, pancreatic) (Fig. 1b, left panel). Furthermore, we tested the effect of B3CD on LNCaP (prostate carcinoma), HUVEC, primary fibroblasts (BJ), and an immortalized trophoblast cell line (HTR-8, first trimester cytotrophoblasts) using the latter two cell lines as controls. B3CD displayed a remarkable difference in cytotoxicity depending on the cell line but not the tissue or tumor origin studied, with an IC₅₀ (24 h of treatment) of ~10 μ M for HTR, LNCaP, BxPC-3 and OVCAR-3 in contrast to an IC₅₀ of ~300 nM for SKOV-3, PC3, BJ fibroblasts and an IC₅₀ between 100 and 300 nM for HUVEC. The cell specific cytotoxicity of B3CD was most imminent at a concentration of 1 μ M with a cell-death of 92% SKOV-3; 90% HUVEC; 88% BJ Fibroblasts; 82% PC-3; 32% OVCAR-3; 30% LNCaP; 18% BxPC-3; 12% HTR (Fig. 1b, left panel). The calcemic parent compound calcitriol/vitamin D₃ (Fig. 1a) did not induce growth-inhibitory effects even at a concentration of 10 μ M in any cell line tested other than LNCaP where some cytotoxicity could be observed at high concentrations (e.g., 10 μ M) (Fig. 1b, right panel).

Morphology changes of ovarian cancer cells after treatment with B3CD or calcitriol/vitamin D₃

To analyze morphological changes of SKOV-3 ovarian cancer cells upon B3CD treatment we carried out light- and fluorescence microscopy after fixation of the cells and staining of the nuclear chromatin with DAPI. In addition, we performed the analysis on fibroblasts as a primary cell line and on HUVEC endothelial cells. The untreated cultures or cells treated with 0.5 μ M of calcitriol/vitamin D₃ for 24 h displayed a homogenous morphology with nuclei lightly and evenly stained by DAPI (Fig. 2a) for all 3 cell lines and the occasional appearance of dividing cells in mitosis (bright blue staining of chromosomes lined up along the metaphase plate; not shown here). In contrast, treatment of cells with B3CD resulted in a drastic change in morphologic features. The majority of SKOV-3 and fibroblasts and all HUVEC cells displayed highly condensed chromatin, and often densely stained nuclear granular bodies ("apoptotic bodies") within fragmented nuclei. All three cell lines, after treatment with 0.5 μ M of B3CD, revealed these classic hallmarks of apoptosis while fibroblasts, in contrast to SKOV-3 and HUVEC, essentially preserved their normal morphological appearance.

Effect of B3CD on proliferation of ovarian cancer cells

As described in the previous sections B3CD is a highly cytotoxic drug for a variety of cell lines. To investigate if B3CD affects the proliferation of SKOV-3 cells or primary cells such as fibroblasts at drug concentrations below 0.1 μ M when viability was partially reduced (Fig. 1), we performed a BrdU incorporation assay. In this colorimetric assay, color intensity at 450 nm correlates directly to the amount of BrdU incorporated into the DNA, which in turn represents proliferation. B3CD dose-dependently reduced SKOV-3 proliferation by 83% (Fig.

2b) at a concentration of 600 nM (see Fig. 1) and also at sub-cytotoxic concentrations of 20 and 60 nM. In contrast, fibroblast proliferation was affected only at 600 nM (by 42%) but not at 20 or 60 nM. Because BrdU incorporation into the DNA of SKOV-3 cells (but not fibroblasts) was reduced even at B3CD concentrations in the low nanomolar range, this drug is not only a potent cytotoxic but also an anti-proliferative agent for this cell line.

Effect of pro-apoptotic p38 MAPK inactivation in SKOV-3 ovarian cancer cells after B3CD treatment

We screened the effect of various inhibitors against mitogen activated protein kinases (MAPK) on B3CD mediated cell death of SKOV-3. Cells were pre-incubated for 2 h with either two different inhibitors against p38 MAPK or inhibitors against Erk 1 (p42 MAPK) and Erk2 (p44 MAPK) or JNK 1, 2 and 3, or with an inhibitor against Jun/JNK or MEK (see Materials and Methods). Derivatives of these inhibitors served as negative controls. SKOV-3 cells were treated with 0.25–3 μ M B3CD in the continued presence of the inhibitors for an additional 24 h and a MTS viability assay was carried out as described (Materials and Methods). In this screen, a dramatic suppression of the cytotoxicity of B3CD was achieved by interfering with the activity of p38 MAPK. Both p38 inhibitors, at a concentration of 20 or 40 μ M (Fig. 3a), could completely counteract the cytotoxicity of B3CD (0.5 μ M). At a B3CD concentration of 1 μ M, the cytotoxic effect could still be reversed by 66% and 76%, respectively. In this screen, inhibitors against Erk1 and 2 had no effects at a concentration of 40 μ M. This is the maximum inhibitor concentration feasible because even in the absence of B3CD higher amounts of both the inhibitor and the inhibitor derivative used as controls displayed unspecific toxicity towards SKOV-3 cells (data not shown). Similarly, the highest non-cytotoxic concentration (10 μ M) of JNK1/2/3 and Jun/JNK inhibitors did not reverse the effect of B3CD treatment, while the MEK inhibitor at 10–30 μ M showed inconsistent effects with occasional marginal to partial reversal of B3CD cytotoxicity in some experiments (data not shown).

We analyzed the expression of p38 MAPK before and after B3CD treatment of SKOV-3 by Western Blot analysis using primary antibodies against pro- and activated/phosphorylated p38. Immunoblotting revealed a high basal expression, a 2–3 fold up-regulation within 5 min following B3CD treatment, and decreasing expression within 1–6 h of B3CD treatment of non-activated p38 MAPK (Fig. 3b, middle panel). As an internal standard for equal loading the blots were probed with an anti-beta-Actin antibody (Fig. 3b, bottom panel). Remarkably, activation/phosphorylation of p38, which in non-treated SKOV-3 is barely detectable, increased ~5–10 fold within 5 min after cell exposure to B3CD and continued over time (Fig. 3b, upper panel). The observation of B3CD induced p38 MAPK activation supports the key finding in viability assays (Fig. 3a) that inhibition of p38 signaling counteracts B3CD induced cell death.

Anti-cancer activity of B3CD in an ovarian cancer xenograft model

The anti-tumor efficacy of B3CD was studied using human ovarian SKOV-3 cancer cell derived xenografts in 4–6 week-old nude (NU/NU) mice. SKOV-3 cells suspended in matrigel were inoculated subcutaneously in one flank of each animal. Mice were treated intraperitoneally every other day with either B3CD (0.15 mg/kg) (mice B1-16; Fig. 4, panels c/d) or vehicle control (mice C1-9; Fig. 4, panels a/b). Animals were weighed (panels b/d) and tumor size calculated (panels a/c) using a caliper every 4–6 days.

Throughout the study period no significant ($\geq 15\%$) weight loss or gain was observed in either group. Tumor size consistently increased in the control animals (Fig. 4a). While several mice were euthanized before the end of the study (C2, C4, C5, C6, C8) because of ulcer formation at the tumor site, no animal in the control group was euthanized due to tumor growth beyond 20 mm in diameter. This observation is in clear contrast to the effect of B3CD on tumor

development (Fig. 4c). Several animals in the treatment group were euthanized secondary to ulcers formation (B8, B10, B12, B14), and because of tumor size ≥ 20 mm (B7, B9, B15). The tumors of these three mice grew from 0 mm, 7.5 mm and 8 mm, respectively, to over 20 mm in diameter in only 4–5 weeks (Fig. 4, panel c). However, the majority of B3CD treated mice at study end revealed delayed tumor growth. In addition, several animals (B5, B13, B16) in the treatment group displayed complete responses (full tumor regression) to B3CD as early as 4 weeks following initiation of treatment. These animals were observed for an additional 14 weeks following completion of treatment and no tumor recurrence was seen. Kaplan-Meier analysis was used to evaluate tumor growth and included all 25 animals at the start of the study while accounting for animals euthanized secondary to skin ulceration. All animals were included in the analysis, i.e., remained in the denominator, until one of the following outcomes occurred: (1) tumor growth surpassed 15 mm in largest diameter (event) or (2) observation ended while tumor was ≥ 15 mm in largest diameter (censored event). Ulceration occurred in several untreated and B3CD treated animals and appeared to not be linked to rapid growth in untreated animals and therefore did not constitute an event included in the analysis. By this method, 100% of the control mice and 40% of the B3CD-treated animals had tumors ≥ 15 mm at the end of the study ($p=0.3$ by log-rank test) (Fig. 5).

In summary (Table 1), all control mice (100%) revealed tumors ≥ 15 mm in diameter at study end, and midway through the study the majority of control animals (83%) displayed tumors between 10–15 mm in diameter and no animals showed tumors ≤ 10 mm. In contrast, at study end, the majority (77%) of B3CD-treated mice displayed tumors ≤ 15 mm in diameter including 33% of animals with tumors ≤ 10 mm. Remarkably, even at the study midpoint, 43% of B3CD-treated mice displayed tumors between 10–15 mm in diameter, 43% displayed tumors ≤ 10 mm, and only 14% displayed tumors ≥ 15 mm.

Discussion

The low survival rate for women suffering from ovarian cancer is related to the development of resistance to platinum-derived chemotherapeutic agents [2,3,5,7,8]. One aspect of the present study was to investigate the cytotoxic effect of B3CD, a derivative of vitamin D₃ precursor calcidiol on human ovarian cancer cell lines in comparison to cell lines derived from other tumors and control cell lines. We chose ovarian cancer cell lines SKOV-3 and OVCAR-3 that are resistant to drugs such as Adriamycin and Cisplatin (ATCC, Manassas, VA; www.atcc.org).

Previous work showed that B3CD at low concentrations displayed growth-inhibitory effects both with respect to proliferation and viability in prostate cancer cell lines [21] and neuroblastoma cell lines [22] while other cancer cells such as MCF-7 (breast cancer) or primary keratinocytes were significantly less affected [17,20,21]. Thus, B3CD in contrast to the parent compound calcitriol/vitamin D₃, which is highly calcemic *in vivo*, was proposed to be a potential anti-cancer drug highly specific for certain cancer types. In the present study B3CD, similar to calcitriol/vitamin D₃ (when used at higher concentrations) and various derivatives [27–30], displayed a 10 to 20-fold difference in cytotoxicity depending on the cell line studied (cytotoxicity at 1 μ M B3CD: SKOV-3 cells > HUVEC > fibroblasts > PC-3 > OVCAR-3 > LNCaP > BxPC-3 > HTR). However, no specificity with respect to tumor origin (ovarian vs. prostate vs. pancreatic cancer cell line), tissue type (adenocarcinoma vs. non-adenocarcinoma) or transformation status (cancer cell vs. primary cell vs. cell line with extended life span) could be observed. The cell line specificity (in contrast to tumor type specificity) is reflected by the two ovarian cancer cell lines studied: SKOV-3 cells, unlike OVCAR-3, were highly sensitive to B3CD treatment. Another example of this phenomenon is the response of the control cells studied. Fibroblasts were 10-times more sensitive to B3CD treatment than trophoblasts. However, our observation that B3CD, at concentrations in the nanomolar range, differentially

affected SKOV-3 cell and fibroblast proliferation (DNA synthesis) suggests that this compound, at sub-cytotoxic concentrations, might possess selective effects on tumor vs. primary cell lines. Thus, B3CD is a promising compound to study the mechanisms of proliferation and survival- vs. cell death-signaling of tumor cell lines.

Cell death of tumor cells as a result of treatment with B3CD is an apoptotic event, as previously shown for neuroblastoma and prostate cancer cell lines [21,22]. Apoptosis in various neuroblastic cell lines is mediated by the intrinsic signaling pathway of apoptosis [22] whereas for prostate cancer cells, in addition, apoptosis is mediated by the extrinsic pathway as shown by analysis of the expression of apoptotic markers, morphological features, and DNA fragmentation [21]. Accordingly, SKOV-3 cells displayed hallmarks of apoptosis after B3CD treatment.

In the present study, an endothelial umbilical vein cell line (HUVEC) was added to the panel of B3CD treated cells. B3CD revealed high cytotoxicity against HUVEC cells comparable only to SKOV-3 cells, while we did not observe any significant effect of the parent compound calcitriol/vitamin D₃ on HUVEC cells at concentrations up to 10 μ M. The latter observation supports previous findings that vitamin D₃ does not affect HUVEC viability or proliferation unless treatment was combined with cytokines such as TNF- α [31]. Following B3CD treatment, HUVEC cells displayed condensed chromatin and nuclear granular bodies (“apoptotic bodies”). While other studies suggested that vitamin D₃ or its precursor calcidiol decreased the proliferation of HUVEC cells and tumor-derived endothelial cells [32–34] our findings suggest that B3CD might be superior to the parent compound for mechanistic studies of angiogenesis.

The present report includes an initial study of the effect of various MAPK inhibitors on B3CD induced cytotoxicity in SKOV-3 cells. P38 and other MAPKs such as JNK, MEK, Erk1/2 mediate signaling pathways in cancer and control cells responding to inflammatory cytokines, UV light, diverse pro-apoptotic stimuli as well as cytotoxic drugs [35–41] such as with Cisplatin in ovarian cancer cells [42]. We show that a dramatic suppression of B3CD cytotoxicity was achieved by interfering with the activity of p38 MAPK while inhibition of Erk 1, Erk2, JNK 1, 2 and 3, Jun/JNK, or MEK did not significantly alter B3CD mediated cell death. Immunoblotting revealed that this drug caused an enhanced expression of inactive p38 MAPK and up-regulation of activity of this pro-apoptotic signaling factor. P38 MAPK appears to play a significant role in the regulation of ovarian cancer by differential and competing effects. Previously, inactivation of p38 function (DN-p38 mutation in SKOV-3 and CaOV-3 by transient transfection) was shown to inhibit EGF-dependent ovarian cancer cell invasiveness [43] and p38 up-regulation has been correlated with increased tumorigenesis yet decreased cell–cell adhesion in an ovarian cancer xenograft model *in vivo* [44]. Moreover, the resistance of IGROV-1 ovarian cancer cells to treatment with platinum drugs and subsequent apoptosis was correlated to insensitivity to EGFR inhibition as well as to an up-regulation of p38 activity [45]. In contrast, p38 activation generally is a pro-apoptotic trigger and in ovarian cancer cells is a key determinant for drug (platinum compound CDDP)-induced apoptosis [46]. Similarly, the use of p38 inhibitors can remarkably suppress the cytotoxicity of non-platinum drugs in ovarian cancer cells [35]. The present study suggests that the mechanism of B3CD induced cell death of SKOV-3 is mediated by the p38 MAPK signaling pathway with other MAPKs either marginally, or not involved. However, as summarized above, p38 MAPK signaling is dependant on factors such as drug sensitivity of the cell line or tumor tissue tested, drug type and concentration applied, the level and duration of p38 activation or other effects such as growth factor (e.g. EGF) sensitivity. Consequently, we suggest studying the dual role of p38 signaling in apoptotic and/or tumorigenic events in ovarian and possibly other cancer cells *in vivo* and *in vitro*. Future studies could include detailed kinetic and dose-dependent responses

of ovarian cancer cells during co-treatment with p38 inhibitors or growth factors (e.g. EGF) up-regulating the expression of p38 MAPK.

Despite the fact that B3CD affected the viability of primary cell lines *in vitro*, a systemic study in SCID (CD-1) mice showed that B3CD did not raise serum calcium levels or induce systemic toxicity at or below concentrations of 166 µg/kg (by IP) [21]. Moreover, bromoacetic acid, a possible metabolite after B3CD administration in clinically relevant doses, is unlikely to reach toxic concentrations: the LD₅₀ of bromoacetic acid in male rats is several hundredfold higher (87.8 mg/kg) [47]. These findings led us to conduct an *in vivo* study in nude (NU/NU) mice applying B3CD (by IP) at a non-toxic concentration of 150 µg/kg (~280 nM) in an ovarian cancer xenograft model. This immunodeficient mouse strain originated from the NIH, lacks a thymus, is unable to produce T-cells, and is used for xenograft, and syngeneic tumor studies. The *in vivo* study of the chemotherapeutic properties of B3CD on human ovarian SKOV-3 cancer cell derived tumor xenografts was conducted over a period of 8 weeks and lead to the following key observations:

- i. Tumor size consistently increased in untreated control animals over the observation period and all control mice had tumors ≥15 mm diameter at study end.
- ii. In contrast, while several animals in the B3CD treatment group were euthanized because of excessive tumor growth (≥20 mm), the majority of treated animals displayed tumors ≤15 mm in diameter, even at study completion. In addition, several treated animals (25%) displayed complete responses to B3CD treatment, and during the extended observation period (up to 14 weeks after the end of treatment), no tumor recurrence was seen.

Thus, B3CD treatment revealed a chemotherapeutic effect in a majority of animals. However, a significant number of animals displayed an accelerated tumor growth likely due to non-specific, and as yet to be identified adverse effects of B3CD in these immunodeficient mice that counteracted the chemotherapeutic effect of this drug. We postulate that the immune status of the animals may have a significant role in tumor growth and response to B3CD. We propose to continue evaluating of this bromoacetoxy-ester derivative of calcidiol in cancer model systems and we are currently developing further non-calcemic bromoacetoxy derivatives of vitamin D₃ as potential anti-cancer drugs [48]. In addition, we suggest mechanistic studies on the signaling effects of B3CD (and the use of inhibitors and/or co-treatment with cytokines), and other vitamin D₃ bromoacetoxy-ester derivatives in tumor cells and on angiogenesis, and their translation into *in vivo* models of tumor development.

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Fig. 1.

Comparative analysis of the cytotoxic effect of B3CD and calcitriol/vitamin D₃ on ovarian cancer and control cell lines. Panel **a**: Structure of Calcitriol, Calcitriol/Vitamin D₃ and derivative B3CD. Panel **b**: Effect of B3CD on cell viability. The cytotoxicity of B3CD and calcitriol/vitamin D₃ in ovarian cancer cell lines (SKOV-3, OVCAR-3) was compared to the effect in pancreatic cancer cells (BxPC-3), prostate cancer cells (PC-3, LNCaP), endothelial cells (HUVECs) and primary fibroblasts (BJ). The cells were treated for a total of 24 h with various drug concentrations (0–10 μM). The MTS viability assay was carried out as described (Materials and Methods). Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations ($X \pm SD$) of a representative experiment in percent cell viability of untreated cells [=100%]

Fig. 2.

Effect of B3CD on morphology and proliferation of ovarian cancer cells and fibroblasts. Panel **a**: Effect of B3CD on cell morphology. Ovarian cancer cells (SKOV-3), endothelial cells (HUVEC), or primary fibroblasts (BJ) were treated for 24 h with B3CD and calcitriol/vitamin D₃ at a concentration of 0.5 μ M before microscopic analysis by phase contrast (PC) or fluorescence analysis after chromatin staining (DAPI) as described (Materials and Methods). Images obtained from a representative experiment are shown. Bar= 10 μ m. Panel **b**: Effect of B3CD on cell proliferation. Ovarian cancer cells (SKOV-3) or primary fibroblasts (BJ) were treated with various concentrations (0–0.6 μ M) of B3CD. A colorimetric assay (based on BrdU incorporation detected by an BrdU-antibody peroxidase conjugate) was carried out as described (Materials and Methods). The color intensity at 450 nm correlates directly to the amount of BrdU incorporated into the DNA, which in turn represents proliferation. Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations ($X \pm SD$) in percent of absorbance by triplicate samples of untreated cells [=100%]

Fig. 3.

Expression and activation of pro-apoptotic MAPK p38 in SKOV-3 cells after B3CD treatment, effect of p38 inactivation on cell viability. Panel **a**: p38 inhibition after B3CD treatment restores cell viability. SKOV-3 cells were pre-incubated with two different specific inhibitors against p38 MAPK (A: SB203580, B: SB202190) or a derivative of these inhibitors as control for 2 h and treated with 0.5 or 1.0 μM B3CD in the continued presence of the inhibitors (40 μM) for an additional 24 h. The MTS viability assay was carried out as described (Materials and Methods). Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations ($X \pm SD$) of a representative experiment in percent cell viability of untreated cells [=100%]. Panel **b**: Activation of p38 MAPK. SKOV-3 cells were treated with 1 μM B3CD for 5 min, 1 h, or 6 h. Western Blot analysis was carried out as described (Materials and Methods) using primary antibodies against pro- and activated/phosphorylated (P-) p38 MAPK. As an internal standard for equal loading the blots were probed with an anti-beta-Actin antibody

Fig. 4.

Anti-cancer activity of B3CD in an ovarian cancer model. SKOV-3 cells were inoculated subcutaneously in one flank of immunodeficient nude mice. After tumor development, mice were treated intraperitoneally every other day with either B3CD (0.15 mg/Kg) (mice B1-16; panels **c**, **d**) or vehicle control (mice C1-9; panels **a**, **b**). Mice were weighed (panels **b**, **d**) and tumor size calculated (panels **a**, **c**) using a caliper every 4–6 days. The length of the study was 8 weeks. Mice were euthanized if tumors were greater than 20 mm in diameter (B7, B9, B15 in treatment group) or if ulcers formed (C2, C4, C5, C6, C8 in control group; B8, B10, B12, B14 in treatment group). There was no loss of $\geq 15\%$ body weight at any time point during the evaluation period. For animals with complete response to treatment (full tumor regression; B5, B13, B16) the observation period was extended by 14 weeks; no tumor recurrence was observed

Fig. 5. Kaplan-Meier analysis of tumor growth in an ovarian cancer animal model. Kaplan-Meier estimates of time until tumor surpassed 15 mm in diameter (by treatment group). Animals that developed ulcers were treated as censored events. P -value=0.3 by log rank test of equality of survival curves

Table 1

Percentage of mice in B3CD treatment group vs. control group with respect to tumor size

Treatment group		Tumor size		
		≤10mm	10–15mm	≥15mm
Controls	Day 0	100%	0%	0%
	Day 24/26	0%	83%	17%
	Day 52/54	0%	0%	100%
B3CD treated	Day 0	100%	0%	0%
	Day 24/26	43%	43%	14%
	Day 52/54 ^a	33%	44%	22%

^a If the three mice that were euthanized (because of tumor size > 20 mm) at earlier time points are included, the percentages would be 33, 25, and 42%, respectively