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Growth Inhibition of Cancer Cells by An Active Metabolite of a Novel Vitamin D Prodrug

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Abstract. *Active vitamin D compounds have been developed that maintain antiproliferative properties with low calcemic activity. BCI-210, a novel vitamin D pro-drug developed in our laboratory, is activated through side chain hydroxylation and possesses lower calcemic activity than calcitriol. The human hepatoma cell line (HepG2) was used to produce an active metabolite, which was characterized and identified as 27-hydroxy-BCI-210. We compared the ability of 27-OH-BCI-210 with calcitriol to inhibit proliferation of prostate (LNCaP), and breast (MCF-7) cancer cells. Cells were plated in multi-well plates and incubated with vehicle or vitamin D compounds for 6 days, after which the cell numbers were determined by a colorimetric assay. 27-OH-BCI-210 produced a dose-dependent growth inhibition, although a concentration five-fold greater than calcitriol was required to produce equivalent inhibition. We also examined the antiproliferative activity of 27-OH-BCI-210 in combination with chemotherapeutic drugs. With genistein and doxorubicin, 27-OH-BCI-210 produced synergistic inhibition of proliferation of LNCaP and MCF-7 cells. These synergistic interactions suggest the potential clinical utility of 27-OH-BCI-210 in the treatment of prostate and breast tumors.*

Vitamin D_3, a secosteroid, produced in the epidermis or obtained from the diet, must be metabolized to the dihydroxylated form (calcitriol, $1,25(OH)_2D_3$) to be biologically active. In addition to its classic functions, the active hormonal form of vitamin D_3 is involved in the regulation of proliferation and differentiation of a variety of normal and cancer cells, including prostate and breast cancer (1,2). This effect is mediated mainly by the nuclear vitamin D receptor (VDR), a ligand-regulated transcription factor in the steroid receptor superfamily (3). Calcitriol has been demonstrated to have antitumour activity both *in vitro* and *in vivo* (4-6) and is currently under investigation as a therapy for several types of malignancies (7,8). The inhibitory role of calcitriol in cellular growth and proliferation potentially provides protection from various types of cancers, as indicated by several epidemiological studies (9,10). However, while calcitriol has been found to be efficacious in several preclinical and clinical studies, dose-limiting calcemic effects have proved a major obstacle to the use of this compound as a chemotherapeutic or chemopreventive agent (11). Therefore, vitamin D analogues are being sought, which exhibit similar or enhanced growth-inhibitory and antimetastatic properties with reduced calcemic toxicity at therapeutic dosages (12).

A common approach to cancer chemotherapy involves simultaneous administration of two or more chemotherapeutic drugs to patient, in the hope that the drug combination will be more effective than any single agent. In the last decade, several studies have been conducted on combination therapy of calcitriol or its analogues with commonly used chemotherapeutic drugs (13-15).Vitamin D compounds and chemotherapeutic agents have been demonstrated to synergistically interact to inhibit the growth of human prostate and breast cancer cells in culture (16,17). An active vitamin D_2 metabolite, $1,24(OH)_2D_2$, has demonstrated synergistic enhancement of growth inhibition in combination with several anticancer drugs at clinically relevant concentrations (18). The antiestrogen tamoxifen has also been shown to act synergistically with vitamin D in the inhibition of breast cancer cell growth *in vitro* (19).

BCI-210, a novel vitamin D analogue developed in our laboratory, is a pro-drug that must be activated through side chain hydroxylation before it can exert biological effects. This molecule possesses a molecular structure similar to $1\alpha(OH)D_2$ (doxercalciferol or Hectorol®), but contains an additional C25-C27 double bond and has inverted stereochemistry at C24 (Figure 1A). BCI-210 has been shown to have activity equivalent to $1\alpha(OH)D_2$ at reducing PTH levels *in vitro*, and to possess 5- to 10-fold lower toxicity relative to doxercalciferol in animal models (unpublished observation).

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It was of interest to determine whether BCI-210 has any antiproliferative activity against cancer cells. To examine this, we generated sufficient quantities of a metabolite of BCI-210 in a HepG2 cell line to analyze its structure and to evaluate its antiproliferative activity. We compared the efficacy of this metabolite with calcitriol and in combination with the chemotherapeutic drugs, doxorubicin and genistein, on prostate and breast cancer cell lines.

Materials and Methods

Cell lines and reagents. Human cell lines hepatoma (HepG2), prostate carcinoma (LNCaP), breast adenocarcinoma (MCF-7), and all cell culture reagents were obtained from the American Type Culture Collection (Rockville, MD, USA). Bovine Serum Albumin (BSA), the antioxidants 1,2-dianilinoethane (DPPD) and all chemotherapeutic agents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Concentrated stocks of doxorubicin and genistein were made from sterile distilled water and DMSO, respectively.

Doxercalciferol ($1\alpha(OH)D_2$), BCI-210, and calcitriol ($1,25(OH)_2D_3$)(Bone Care International) were dissolved in 100% ethanol and stored, protected from light, as concentrated solutions at –70˚C.

Cell culture. HepG2 and MCF-7 cells were maintained in DMEM medium, whereas RPMI-1640 was used for the culture of LNCaP cells. The medium of both types were supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Stock cultures were maintained in 100-mm^2 dishes and incubated at 37˚C in a humidified atmosphere of 5% CO_2. Cell populations were passed weekly with the medium replaced every 2-3 days after passage.

Metabolite production. Metabolites of BCI-210 were generated as previously described (20). Briefly, HepG2 cells were subcultured in 100-mm^2 plates and grown to confluence in serum-containing medium. Before incubation, plates were washed with PBS and re-fed with 4 ml of serum-free medium containing 1% BSA and 100 μM DPPD. BCI-210 was then added to the HepG2 cell cultures at a final concentration of 50 μM and the culture incubated for 48 hours.

Following incubation, the medium was transferred to glass tubes and the cells were washed with an equal volume of methanol. The medium and methanol were then combined and lipids extracted using a modification of the method of Bligh and Dyer (21), in which chloroform was replaced by methylene chloride. The organic phase was recovered, dried under nitrogen, and the residue redissolved in mobile phase and subjected to high-performance liquid chromatography (HPLC). The extracted metabolites were separated on a normal phase column (Agilent Zorbax SIL 5 μM, 4.6 mm x 150 mm) and eluted under isocratic conditions using a mobile phase of hexane/isopropyl alcohol (HX/IPA) (90:10, v/v), at a flow rate of 2.0 ml/min at 30˚C. Vitamin D metabolites were monitored by measuring absorbance at 265 nm. The metabolite was considered sufficiently pure for NMR analysis when a single peak was observed on HPLC after repeated purification. The samples were stored in amber vials at –20˚C prior to their use.

NMR. NMR analysis was performed using 500 MHz proton frequency and a 5-mm Shigemi NMR tube on a Varian Unity-Inova NMR spectrometer. BCI-210 (parent compound) or HPLC purified metabolite was dried under nitrogen, mixed with Chloroform-d (Sigma-Aldrich 100.0 atom % D) and then added to the cavity of a Shigemi NMR glass insert (CMS-005TV).

Growth inhibition assays. LNCaP or MCF-7 cells were harvested at confluence and plated into 96-well tissue culture plates at the following densities: LNCaP, 2 X 10^3 cells/well and MCF-7, 5 X 10^2 cells/well. Cells were seeded in triplicate, in a volume of 0.15 ml medium containing 10% fetal bovine serum (FBS) and allowed to grow for 24 h. The plating medium was removed and replaced with 0.1 ml medium containing 10% FBS and vehicle, vitamin D compounds ($1,25(OH)_2D_3$, BCI-210, or metabolite of BCI-210), and/or a chemotherapeutic agent (doxorubicin or genistein). The cells were allowed to grow for an additional 6 days, with all solutions changed on day 3 to maintain a fresh supply of medium nutrients and drugs. To quantitate cell viability, 20 μl of CellTiter 96 Aqueous One Solution Assay Reagent (Promega Corp., Madison, WI, USA) was added to each well, and the plates were incubated for 90 min at 37˚C in a humidified atmosphere of 5% CO_2. Absorbance was read with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 490 nm. Growth inhibition was calculated as the percent difference of the treated cells compared with the vehicle control. Each experiment was carried out in triplicate.

Isobologram analysis of combination treatments. Isobologram analysis was performed using the CalcuSyn software program (Biosoft, Cambridge, UK). Data from growth inhibition assays for individual drugs, as well as combinations of the two drugs, were entered into the program to produce median-effect plots from which isobolograms were constructed. The accurate prediction of drug interactions is not possible from the simple addition of individual drug effects because of the non-linearity of the response curve for each drug (22). The isobologram method of analysis alleviates this problem by evaluating drug interaction independent of the shape of the dose response curve, and allows for concise classification of the degree of interaction between two drugs (23). The analysis is based on the median-effect principle and the median effect equation (24,25).

Statistical analysis. All results are expressed as mean±SEM. One-way analysis of variance (ANOVA) and non-paired *t*-test were used to compare the effect of the chemotherapeutic drugs alone combined with vitamin D compounds.

Results

Identification of metabolites of BCI-210 in HepG2 cells. The structure of BCI-210 is shown in Figure 1A. BCI-210, at concentrations between 25 μM and 125 μM, was incubated with HepG2 cells in order to determine the optimal concentration for metabolites production. The optimal substrate concentration was determined to be 50 μM; higher concentrations proved toxic to the cells. The principal metabolite of BCI-210 generated in HepG2 cells migrated with a retention time of 8.163 minutes, Figure 1B. Similar results were observed for metabolites that were generated from Hep3B cells (data not shown).

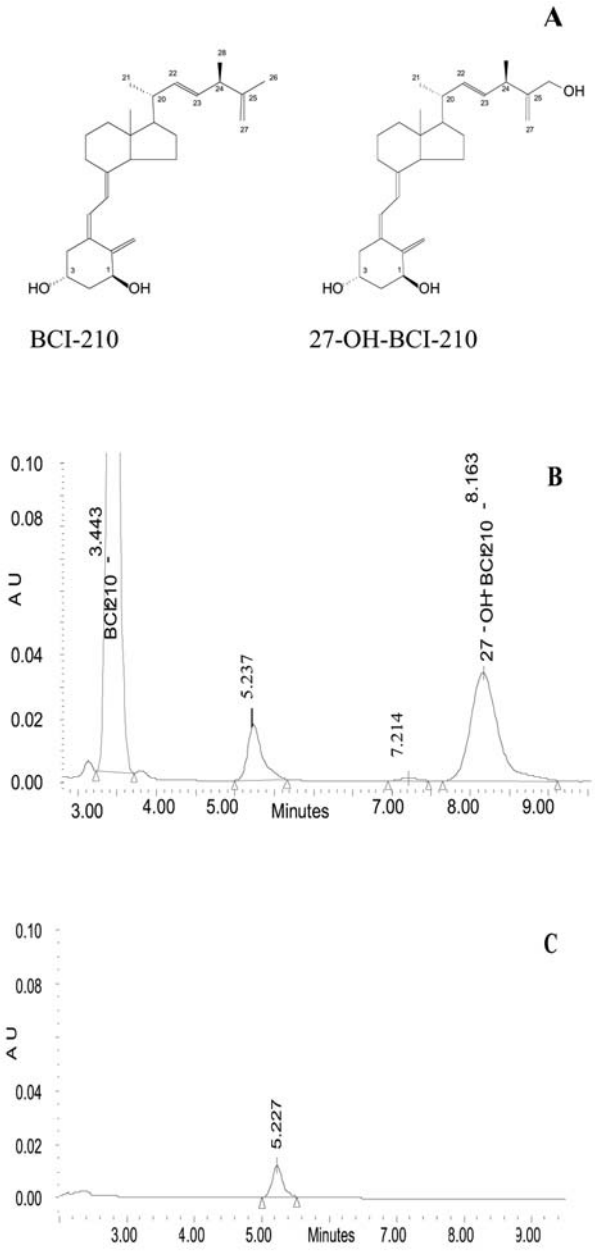

Figure 1. *Structure and HPLC profile. Pro-drug BCI-210 was incubated with HepG2 cells for 48 hours at 50 μM concentration. The cells were extracted and purified by UV spectra and HPLC. A is the structure of BCI-210 and 27-OH-BCI-210. B is the HPLC profile of BCI-210 and its metabolite. C is the control incubated with ethanol only.*

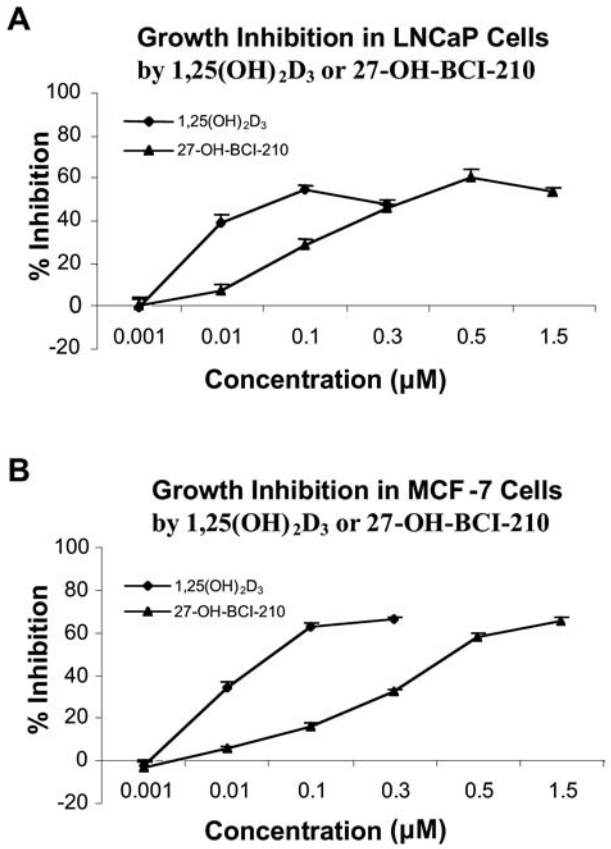

Figure 2. *Dose-dependent effects of 1,25(OH)$_2$D$_3$ or 27-OH-BCI-210 alone in LNCaP (A) and MCF-7 (B) cells in vitro. LNCaP and MCF-7 cells were plated into 96-well plates and incubated with the indicated doses of 1,25(OH)$_2$D$_3$ or 27-OH-BCI-210. Growth inhibition was calculated on day 6 as the percent difference between the drug-treated cells and vehicle control cells. Curves indicate the mean±standard error of six data points per treatment.*

The structure of the major metabolite of BCI-210, 27-OH-BCI-210, is shown in Figure 1A. This metabolite of BCI-210 was characterized by UV absorption profile, HPLC and NMR spectroscopy (26). The NMR spectra of the metabolite was compared with the parent compound, leading to the conclusion that the metabolite had been hydroxylated at the C-27 position to produce the metabolite 27-OH-BCI-210 (Figure1B). Figure 1C shows the results of incubating HepG2 cells with ethanol only.

Antiproliferative activity of 27-OH-BCI-210 and 1,25(OH)$_2$D$_3$. Preliminary experiments were carried out to determine the concentration that would elicit an inhibitory growth response in LNCaP and MCF-7 cancer cells. Treatment with 1,25(OH)$_2$D$_3$ over a dose range of 0.001 μM to 0.3 μM produced maximal growth inhibitory effects at the 0.1 μM dose level of 54.8±1.6% and 62.8±1.8% (mean±SE) for LNCaP and MCF-7, respectively. Increasing the concentration of 1,25(OH)$_2$D$_3$ to 0.3 μM failed to substantially increase the inhibitory effect on LNCaP cells, and produced only a moderate increase on the MCF-7 cells. Treatment with 27-OH-BCI-210 at a dose of 0.001 μM to 1.5 μM produced a

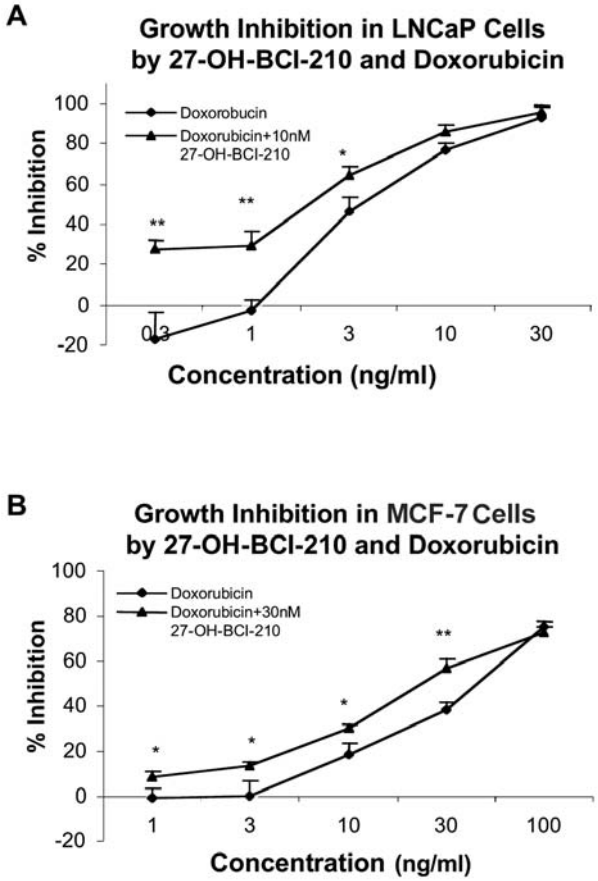

Figure 3. *Effects of the combination of doxorubicin and 27-OH-BCI-210 on LNCaP and MCF-7 cells on growth inhibition. LNCaP (A) and MCF-7 (B) cells were dosed with doxorubicin (ng/ml) and 27-OH-BCI-210 (nM) at the indicated combinations to obtain the growth inhibition curves. Cells were plated in multi-well plates and allowed to grow for 24 hours. The medium was removed and replaced with fresh medium containing vehicle or the vitamin D compounds for 6 days; cell number was determined by a colorimetric assay. Data are expressed as mean±standard error of six wells per treatment. *p<0.05, **p<0.01 indicates significant differences between doxorubicin alone vs. combination treatment.*

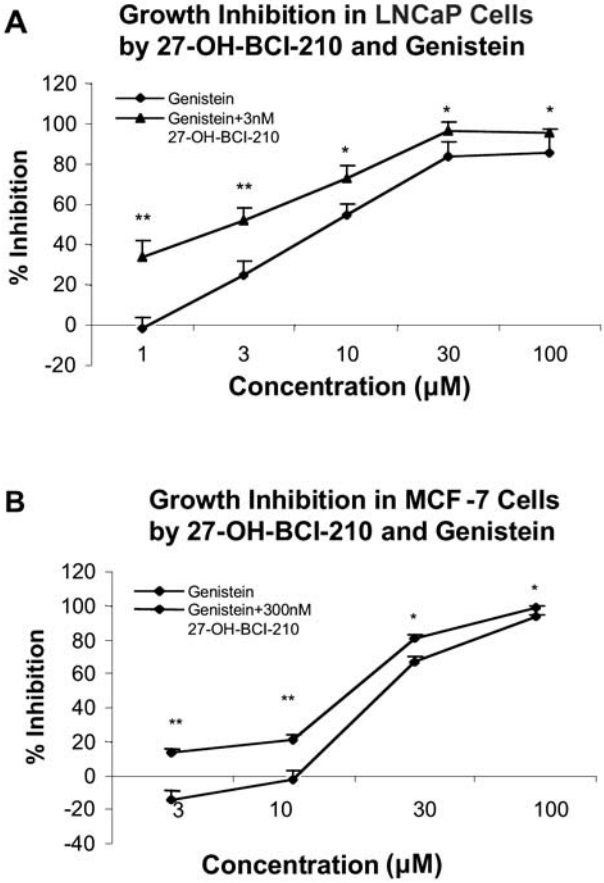

Figure 4. *Effects of the combination of genistein and 27-OH-BCI-210 on growth inhibition of LNCaP and MCF-7 cells. LNCaP (A) and MCF-7 (B) cells were dosed with genistein (μM) and 27-OH-BCI-210 (nM) at the indicated combinations to obtain the growth inhibition curves. The medium was removed and replaced with fresh medium containing vehicle or the vitamin D compounds for 6 days; cell number was determined by a colorimetric assay. Data are expressed as mean±standard error of six wells per treatment. *p<0.05, **p<0.01 indicates significant differences between genistein alone vs. combination treatment.*

maximal inhibitory effect of 60.5±3.4% and 65.4±1.3% in LNCaP and MCF-7 cells, respectively. As seen in Figure 2, 27-OH-BCI-210 produced dose-dependent growth inhibition curves similar to $1,25(OH)_2D_3$ in both LNCaP and MCF-7 strains, although 27-OH-BCI-210 required a 5-fold greater concentration than calcitriol to produce equivalent inhibition.

Combination treatment of 27-OH-BCI-210 and chemotherapeutic drugs. To evaluate the effect of combinations of 27-OH-BCI-210 and chemotherapeutic agents on growth inhibition, cells were incubated with various concentrations of doxorubicin or genistein in the presence or absence of 27-OH-BCI-210. The doses of doxorubicin or genistein to be tested were selected on the basis of previous reports (27). In the combination experiments, doxorubicin concentrations ranged from 0.3 - 30 ng/ml for LNCaP cells and 1-100 ng/ml for MCF-7 cells, whereas the genistein concentration ranged from 1-100 μM for LNCaP cells and 3 - 100 μM for MCF-7 cells. 27-OH-BCI-210 was added at a constant concentration (Figures 3-4).

Figure 3 shows that the addition of 10 nM (A) and 30 nM (B) 27-OH-BCI-210 significantly enhanced the growth inhibition of LNCaP and MCF-7 cells over that obtained by doxorubicin alone at all concentrations except those at the highest dose levels.

Figure 4 shows that the addition of 3 nM (A) and 300 nM (B) 27-OH-BCI-210 enhanced the genistein-induced inhibition of growth at all genistein concentrations. All

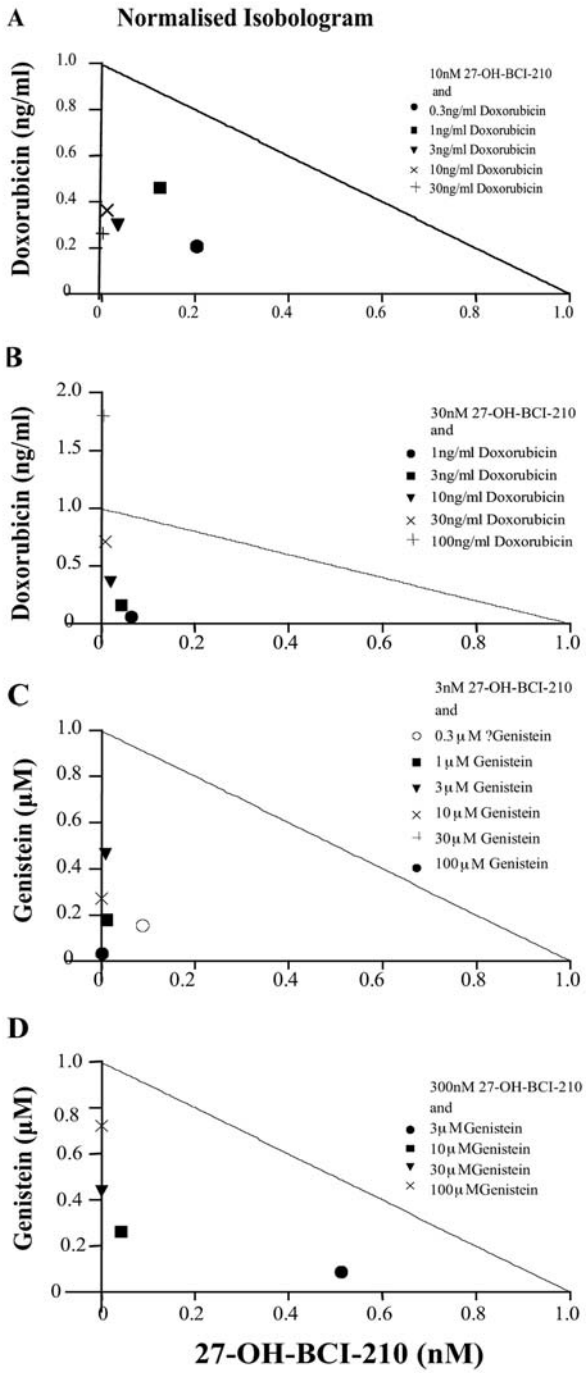

Figure 5. *Synergistic interactions between doxorubicin or genistein and 27-OH-BCI-210 in LNCaP (A & C) and MCF-7 (B & D) cells. Data from growth inhibition assays for individual drugs, as well as combinations of the two drugs, were entered into the software program Calcusyn to produce normalized isobolograms that represent equipotent combinations of compounds at the effective dose of 50% growth inhibition. Individual data points represent the specific dose of doxorubicin or genistein (concentrations indicated on the graph) to the fixed concentration of 27-OH-BCI-210. If a combination data point falls on the diagonal, an additive effect is indicated; on the lower left, synergy is indicated; on the upper right, antagonism is indicated.*

combinations showed a significantly increased growth inhibition effect of 27-OH-BCI-210 over that of genistein alone in both LNCaP and MCF-7. This combination experiment was repeated with four different concentrations of 27-OH-BCI-210 (1, 3, 10, 30 nM for LNCaP cells or 10, 30, 100, 300 nM for MCF-7 cells), all with similar results (data not shown). These results, identifying enhanced antiproliferation action through the combination of 27-OH-BCI-210 plus doxorubicin or genistein in both human prostate carcinoma and human breast adenocarcinoma, indicate that these effects are not cell type specific.

Analysis for synergism. Isobologram analysis (21) was used to identify synergistic interactions between doxorubicin or genistein and 27-OH-BCI-210. MCF-7 and LNCaP cells were treated with several concentrations of doxorubicin or genistein alone or with a fixed dose of 27-OH-BCI-210. The resultant growth inhibition data for individual drugs, as well as combinations of the two drugs, were then entered into the program Calcusyn from which normalized isobolograms were constructed. If a combination data point falls on the diagonal, an additive effect is indicated; under the diagonal, synergy is indicated; above the diagonal, antagonism is indicated. The isobolograms shown in Figure 5 reveal significant synergy between 27-OH-BCI-210 with doxorubicin and genistein at several different combination concentrations. The majority of data points for both drugs indicate that decreasing the dose ratio of doxorubicin or genistein to that of 27-OH-BCI-210 produces a trend toward more synergistic responses as indicated by the points falling to the lower left of the diagonal. Therefore, the combination of nanomolar doses of 27-OH-BCI-210 with low doses of doxorubicin and genistein at these specific dose ratios appears to produce the largest synergistic effects.

Discussion

$1,25(OH)_2D_3$ and its analogues have been shown to inhibit the growth of certain prostate cancer cell lines (28) and inhibit cell proliferation and promote the differentiation of breast cancer cells (29). These results suggested a therapeutic potential of vitamin D compounds in the treatment of prostate and breast cancer. The results presented in the current study show that 27-OH-BCI-210 produces dose-dependent growth inhibition in both LNCaP and MCF-7 cancer cells, although greater concentrations of 27-OH-BCI-210 were required to produce results equivalent to calcitriol.

The potential for the therapeutic use of vitamin D compounds in an oncology setting depends largely on their safety profile upon chronic exposure. Although several less

calcemic vitamin D analogues have been developed as alternatives to calcitriol, hypercalcemia has remained a concern. One possible way to lower the dose of vitamin D compounds and minimize calcemic toxicity is to administer these agent in combination with existing chemotherapeutic drugs that act together to produce a synergistic effect on tumor growth (9). Several studies have identified synergistic interactions between calcitriol and common anticancer drugs such as retinoic acid or docetaxel (15,31). Such combination therapy potentially offers a therapeutic advantage over a standard dose of a single drug, either enhancing the effectiveness of the therapy and/or reducing the dosages of drugs required, thus limiting toxic side-effects. A procedure broadly accepted for distinguishing drug interactions as synergistic or antagonistic is the isobologram method. Using this approach, our results indicate that 27-OH-BCI-210 interacts synergistically with doxorubicin or genistein at several concentrations, thus suggesting that these agents could work well together in cancer patients.

Our study also included that at higher doses of 27-OH-BCI-210 and doxorubicin or genistein, additive effects, as well as one antagonistic response, were possible. This finding is in agreement with similar studies using $1,25(OH)_2D_3$, cisplatin and carboplatin (32), which identified additive or antagonistic effects at higher dose levels. Although the mechanism for this antagonistic effect is unknown, it is well established that the synergy or antagonism between two drugs with different modes of action is largely dependent upon the drug concentrations and ratios. These findings suggest that certain fixed doses of a chemotherapeutic agent, in combination with 27-OH-BCI-210, may improve the anti-tumour response *in vivo*.

Future studies in animal models will be necessary to confirm a potential chemotherapeutic advantage of these combinations and to define the mechanism by which 27-OH-BCI-210 elicits its growth inhibition on prostate and breast cancers. Selection of an appropriately safe and effective compound, for application either as a single or combination treatment, will require a study that directly compares these paradigms. Nonetheless, the current findings indicate the potential usefulness of 27-OH-BCI-210 or BCI-210 for the treatment of cancer.

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