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Inhibition of breast and ovarian carcinoma cell growth by 1,25-dihydroxyvitamin D_3 combined with retinoic acid or dexamethasone

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This study examined the growth inhibitory effects of combining 1,25-dihydroxyvitamin D_3 (calcitriol) with retinoic acid or dexamethasone against cultured breast and ovarian carcinoma cells. Retinoic acid (12.5–50 nM) increased the effectiveness of calcitriol (12.5–50 nM) against MCF-7 and NIH:OVCAR3 cells, with synergistic interactions at two of the three ratios tested. Dexamethasone augmented calcitriol effects, with synergism at 0.05 and 0.1 nM dexamethasone in MCF-7 cells and 5 nM in Caov-4 ovarian cells. This study showed favorable interactions for calcitriol–retinoic acid and calcitriol–dexamethasone combinations in breast and ovarian cancer cell lines.

Key words: Breast neoplasms, calcitriol, dexamethasone, isobolograms, ovarian neoplasms, retinoic acid.

Introduction

Carcinomas of female reproductive tissues are a leading cause of death among women in the US. Breast cancer strikes 200 000 women per year and is a leading cause of female mortality.[1] Ovarian cancer claims 12 000 lives per year and is the most lethal of diseases that affect solely women.[1] Frequently, these cancers become unresponsive to standard therapeutic regimens. In addition, the cytotoxic agents commonly used to treat breast and ovarian malignancies cause significant morbidity. There is a clear need for new therapies with which to treat these two malignancies. An alternative to conventional cytotoxic therapy is cytostatic hormonal therapy, with the goal being to prevent cancer cell proliferation while reducing side effects below the levels associated with conventional cytotoxic therapy. One successful application of this strategy is the utilization of the antiestrogen, tamoxifen, to treat breast cancer in patients whose tumors are estrogen receptor positive. Tamoxifen reduced breast cancer recurrence by 25% and improved survival.[2] However, this still leaves many thousands of breast cancer patients without an established, alternative regimen of hormonal therapy for their disease. Even though 53–61% of ovarian neoplasms are estrogen receptor positive and 28–40% of these neoplasms are progesterone receptor positive, the response of this disease to antiestrogens and progestins has been poor.[3–6] In fact, there presently exists no viable hormonal therapy options for ovarian cancer patients. Vitamin D analogs may be a possible hormonal alternative for breast and ovarian carcinomas as a number of malignancies have been shown to respond to these agents in experimental systems.[7]

Breast cancers possess vitamin D receptors and are growth inhibited by vitamin D analogs both *in vitro* and *in vivo*.[8] Ovarian neoplasms obtained from patients were shown to have specific receptors for vitamin D.[9] Several ovarian carcinoma cell lines are substantially growth inhibited by 1,25-dihydroxyvitamin D_3 (calcitriol), the most active naturally occurring metabolite of vitamin D_3 (unpublished observations, this laboratory and ref. 9). Since combination chemotherapy typically is more efficacious than single-agent therapy, the present study was undertaken to determine if combining calcitriol with other 'hormonal' agents would be more effective than treatment with calcitriol alone in limiting the growth of breast and ovarian carcinoma cells. Retinoids are receiving considerable attention for their chemopreventative effects against breast cancer. Retinoic acid, the most active naturally occurring vitamin A metabolite, was selected as one agent to combine with calcitriol. Retinoic acid was previously shown to inhibit the proliferation of ovarian and breast carcinoma cells.[10,11] Supporting data from this laboratory showed inhibition of a second ovarian carcinoma line, NIH:OVCAR3, by retinoic acid.[12]

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Importantly, calcitriol interacted favorably with retinoic acid in another *in vitro* breast cancer model.[13] Dexamethasone, a potent synthetic glucocorticoid, was selected as another 'hormonal' agent to combine with calcitriol. Both ovarian and breast cancer cells lines have been shown to be growth inhibited by dexamethasone.[12,14] Treatment of breast cancer patients with glucocorticoids resulted in a 15% response rate.[15] Dexamethasone has been used in the Cooper breast cancer therapy regimen for over 20 years,[16] but is has received very minimal attention as an agent for ovarian carcinomas.

The primary goal of the experiments in the current study was to identify conditions which yielded highly favorable interactions between calcitriol and dexamethasone or retinoic acid. The breast cancer and ovarian cancer cell lines selected for this investigation have qualitatively similar responses to calcitriol, retinoic acid and dexamethasone (unpublished data, this laboratory), thus it was expedient to evaluate both cell types in the same study. The results from this study will serve as a basis (drug ratios and concentrations) for later, *in vivo*, analyses, which will be more predictive of clinical responses to these calcitriol-based combinations. Subsequent mechanistic investigations are likely to be expedited using the concentration–response profiles to the drug combinations which were generated in this study.

Isobolographic analysis[17–19] was used in this study to statistically categorize the interactions between the combined drugs. This method readily facilitates analyses of multiple concentrations and ratios of drugs, and mitigates inflated estimates of positive interactions that may be obtained when drugs with non-linear dose–response relationships are combined.[18,19] Data are presented that show favorable interactions for combinations of calcitriol with retinoic acid or dexamethasone in ovarian and breast cancer cell lines.

Materials and methods

Cells

NIH:OVCAR3 (purchased from ATCC, Bethesda, MD) and MCF-7 (gift of Dr Herbert Soule, Michigan Cancer Foundation, Detroit, MI) are, respectively, epithelial carcinoma lines of the ovary and breast. Both are grown inhibited by calcitriol, dexamethasone, and retinoic acid (refs 9–12 and unpublished data from this laboratory). Caov-4 is an epithelial ovarian carcinoma (obtained from ATCC) that is growth inhibited by calcitriol and dexamethasone (unpublished observations, this laboratory). The cells were grown as described previously.[9–12] Dextran–charcoal treatment of calf serum was utilized to remove endogeneous steroids in experiments where the effect of calcitriol on MCF-7 cells was evaluated. The serum was stripped using a modified version of the procedure published by Wiese *et al.*[20] and consisted of three consecutive 60 min exposures to dextran–charcoal at room temperature, followed by sterile filtration and storage at −20°C until use.

Drugs and exposure

Calcitriol was purchased from Duphar (Amsterdam, Netherlands). Retinoic acid and dexamethasone were purchased from Sigma (St Louis, MO). All drugs were dissolved in absolute ethanol, stored at −20°C and sequestered from light exposure. Cells, previously transferred to 24-well plates (Corning, from Baxter Scientific), were exposed 3 days to the drugs, administered alone and in combination. In selecting the drug/hormone concentrations for isobolographic analyses we strove to identify concentrations that would produce the largest gradation of effects and the strongest positive interactions between the drug pairs. The most inhibitory concentrations of some hormones were not used here for that reason. The concentration range for each drug typically ranged from approximately its IC_5 (concentration that causes 5% inhibition of cell growth) to a high concentration ranging between the agent's IC_{25} and IC_{70} in the cell type being tested. These drug concentrations meeting the above criteria were empirically determined from experiments (not shown) carried prior to undertaking the present investigation. The drugs were varied as 3-fold dilutions from the highest concentration used to the lowest, except for dexamethasone which required a broader concentration range. This laboratory's previous experience with drug combinations involving hormonal agents has shown this exposure scheme to be the most likely to identify conditions giving synergism (ref. 21 and unpublished observations).

Determination of growth inhibition

After incubation (in quadruplicate) with drugs, growth effects (cell numbers) were determined by lysing cells with 1.1% glacial acetic acid, 5% ethyl-

hexadecyldimethylammonium bromide in H_2O and counting nuclei with a Coulter Counter model ZM, as we have previously published.[21]

Isobolographic analysis for the interaction of binary drug combinations. The isobolographic method of analysis allows for rigorous classification of the degree of interaction between two drugs into one of five categories within a given range of drug concentrations and molar ratios by graphically comparing IC values (e.g. IC_{20}) with corresponding 95% confidence limits of single agents against IC values and 95% limits of drug combinations.[19,21,22] Isobolographic anlaysis was carried out as described previously.[19,21,22] IC_{20} and 95% confidence limits for singly administered drugs were plotted on the *x*- and *y*-axes and connected by lines to delineate a region representing additive effects. IC_{20}s were used for isobolographic analysis of all experiments except in Figure 3, where the larger overall response of MCF-7 cells to calcitriol and dexamethasone permitted the use of IC_{40}s. If the IC_{20} and 95% confidence limits of the drug combinations fell below this region it was indicative of synergistic interactions. Data points that fell above the delineated region reflected less-than-additive effects, which may be divided into infra-additive, non-interactive or antagonistic classifications.[21,22]

Results

The purpose of these experiments was to determine whether retinoic acid or dexamethasone would enhance the growth-inhibitory effects of calcitriol on cultured breast and ovarian carcinoma cells, and to determine the strength and direction of the interactions between calcitriol and each of the other agents. For each drug combination the data were plotted simply as means and standard errors versus drug concentration to give a general indication of the combined effects and the variability of the results. Then, data from a subsequent, similar experiment were analyzed isobolographically to classify the degree of drug interactions, the interaction of various ratios and/or concentrations, and to provide graphic statistical analyses of the degrees of interaction.

Combined effects of calcitriol and retinoic acid

The first experiment examined the interaction of calcitriol and retinoic acid when applied to MCF-7 breast carcinoma cells. In order to increase the sensitivity of MCF-7 cells to calcitriol, cells were incubated in media depleted of steroids by dextran–charcoal treatment of the calf serum. MCF-7 cells appear to respond to low levels of calcitriol and data suggest that the response is masked by the presence of calcitriol in serum not treated with dextran–charcoal (ref. 9 and unpublished observations this laboratory). For reasons that are not clear, dextran–charcoal stripping of calf serum did not improve the response of OVCAR3 or Caov-4 cells to calcitriol (data not shown), so experiments with these two cell lines were carried out using whole serum. Dextran–charcoal treatment of calf serum did not effect retinoic acid responsiveness of OVCAR3 or MCF-7 cells (data not shown). Figure 1(A) shows that 12.5, 25 or 50 nM retinoic acid all markedly increased the effectiveness of the two lower calcitriol concentrations against MCF-7 cells. When retinoic acid was added to the highest concentration of calcitriol (50 nM), negligible enhancement was observed (Figure 1A). All three levels of retinoic acid caused a very similar enhancement of calcitriol's growth-inhibitory effects. Use of broader retinoic acid concentrations, in combination with calcitriol, did not produce further gradation of the retinoic acid-dependent growth-inhibitory effects (data not shown). This experiment established incubation conditions under which calcitriol and retinoic acid interacted effectively in MCF-7 cells. This information was then utilized to carry out a subsequent isobolographic experiment to determine the degree to which the two drugs interacted. The IC_{20} values and 95% confidence limits for singly administered calcitriol and retinoic acid were 26 ± 4 and 24 ± 5 nM, values that were used to construct the region of additivity in Figure 1(B). For a 1:1 fixed ratio incubation the IC_{20}s were 4 ± 1.5 nM with respect to calcitriol and 4 ± 1.5 nM with respect to retinoic acid (Figure 1B). When plotted, these values lay below the (shaded) region of additivity and indicated synergism between calcitriol and retinoic acid at a 1:1 fixed ratio (Figure 1B). The data point for the 1:2 calcitriol–retinoic acid ratio incubation also lay below the shaded region and reflected a comparable level of synergism to that observed at the 1:1 ratio (Figure 1B). The IC_{20} data point and 95% confidence limits for calcitriol and retinoic acid at the 2:1 ratio lay in the region of non-interaction, a result which suggests that retinoic acid did not enhance the effects of calcitriol at this ratio (Figure 1B).

In the next experiment the effects of combining multiple concentrations of calcitriol and retinoic

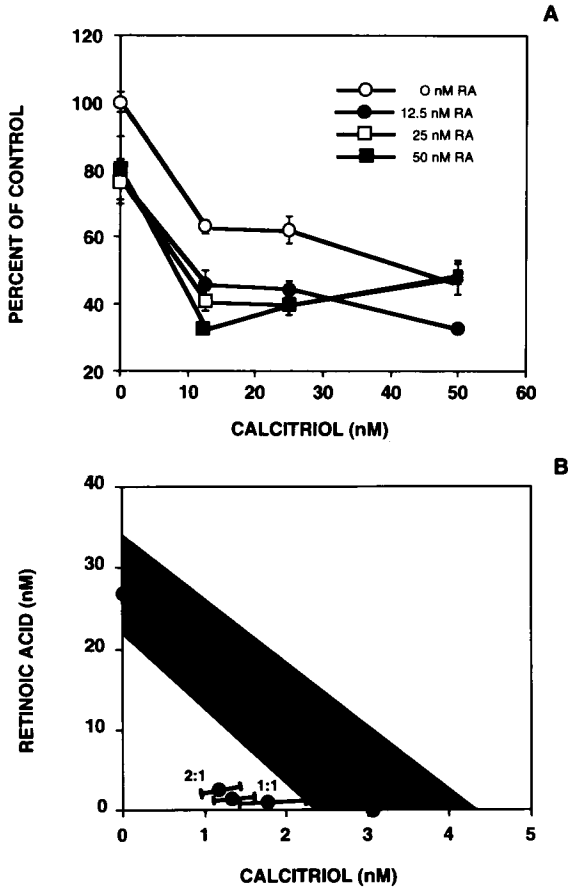

Figure 1. Combined effects of calcitriol and retinoic acid on MCF-7 breast carcinoma cell growth. Cells (seeded at 8000 per well) were incubated 3 days with calcitriol and retinoic acid at multiple levels, indicated as 'RA' in the inset in (A). (A) Growth effects were expressed as mean percent of control cell numbers ± SE for all data points. The *y*-axis was truncated to more clearly indicate the relative effects of each calcitriol concentration. (B) Isobolographic analysis of calcitriol and RA interactions in MCF-7 cells. Using concentration ranges similar to those in (A), cells were exposed to calcitriol and retinoic acid at fixed ratios of 2:1, 1:1 and 1:2 (ratios indicated in the figure). The combined-agent IC_{20} values were determined by curve fitting of response data for each fixed ratio and plotted as points with 95% confidence limits.

acid on the growth of OVCAR3 cells were examined. Retinoic acid, at all levels examined, substantially enhanced the growth inhibitory effects of 12.5 and 25 nM calcitriol (Figure 2A). Retinoic acid caused negligible improvement in the effects of 50 nM calcitriol on OVCAR3 cells (Figure 2A). All three concentrations of retinoic acid caused similar enhancement of calcitriol's effects; however, combinations with broader levels of retinoic acid did not produce any gradation in effects (data not shown). Isobolographic analysis showed that combining retinoic acid (0–50 nM) with calcitriol (0–50 nM) at 2:1 and 1:1 ratios caused the data points for the drug combinations to be significantly below the additivity region, a result which indicated synergism at these ratios (Figure 2B). The interaction of a 1:2 mixture of calcitriol and retinoic acid was borderline between additivity and synergism (Figure 2B).

Combined effects of calcitriol and dexamethasone

In this experiment the enhancement of calcitriol's inhibitory effects by dexamethasone, on the growth

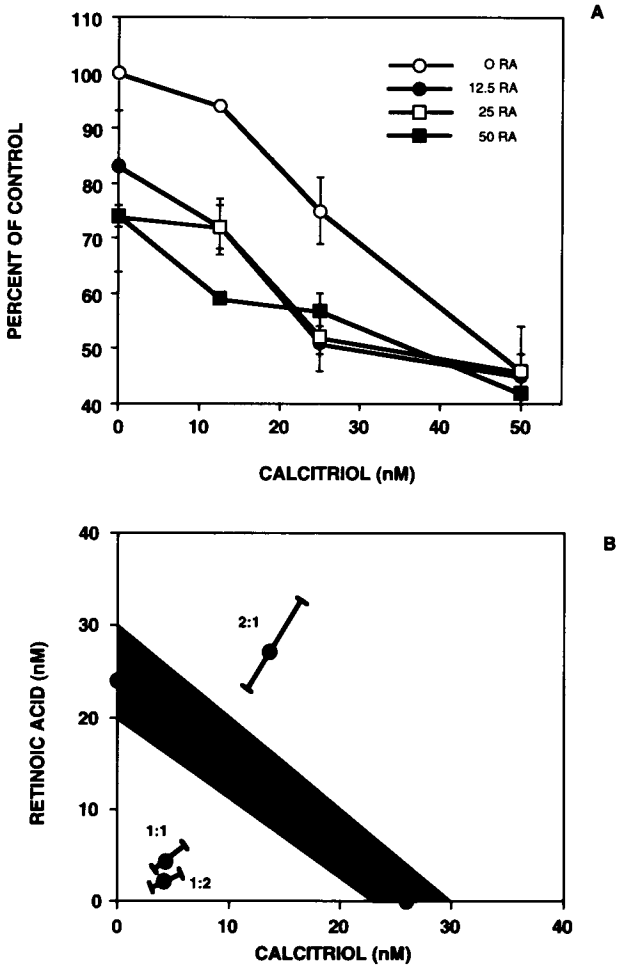

Figure 2. Combined effects of calcitriol and retinoic acid on OVCAR3 ovarian carcinoma cell growth. Cells were incubated and counted as described in the Figure 1 legend. Retinoic acid concentrations are given in the inset in (A). (A) Means ± SE values expressed as percent of control. (B) Isobolographic analysis of calcitriol and RA interactions in OVCAR3 cells. Retinoic acid–calcitriol interactions were evaluated using IC_{20}s.

of MCF-7 breast cancer cells, was probed. Addition of 0–10 nM dexamethasone to calcitriol caused a concentration-dependent enhancement of calcitriol's inhibitory effects against MCF-7 cell growth (Figure 3A), with > 85% growth inhibition occurring when 10 nM dexamethasone was added to all calcitriol concentrations (Figure 3A). The dexamethasone concentration range required to obtain a graded effect was unusually broad for a steroid (or steroid agonist) and precluded the use of fixed-ratio incubations for calcitriol–dexamethasone combinations. Therefore, isobolographic analyses were carried out keeping dexamethasone concentrations fixed, at 0.05 and 0.1 nM, while varying calcitriol concentrations (Figure 3B). The isobologram in Figure 3(B) shows synergistic interactions between varying calcitriol and constant dexamethasone at both 0.05 and 0.1 nM. Dexamethasone at 10 nM could not be examined because it lies outside of the isobol and thus would yield indeterminate results.

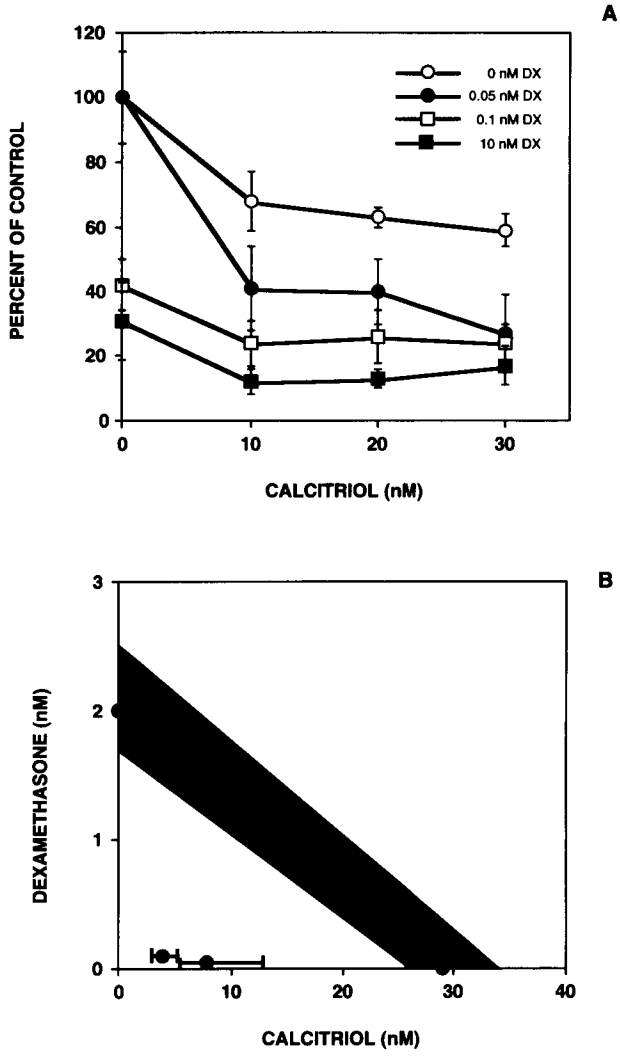

Figure 3. Combined effects of dexamethasone and calcitriol on MCF-7 growth. Cells were incubated and growth effects determined as in Figure 1 except dexamethasone was mixed with calcitriol instead of retinoic acid. Dexamethasone concentrations (DX) are given in the inset in (A). (A) Means ± SE. (B) Isobologram with constant dexamethasone (0.05 and 0.1 nM) and varying calcitriol. Dexamethasone–calcitriol interactions were evaluated using IC_{40}s.

Figure 4 shows the results of combining calcitriol and dexamethasone in ovarian carcinoma cells. These drugs were initially evaluated using OVCAR3 cells. Those data (not shown) suggested favorable interactions between calcitriol and dexamethasone, but were not sufficiently reproducible to permit conclusive evaluation of the results and thus are not presented in this manuscript. Alternatively, calcitriol–dexamethasone interactions were analyzed using Caov-4 ovarian carcinoma cells. The data in Figure 4(A) show that addition of 0.5 nM dexamethasone caused a modest enhancement of calcitriol-induced growth inhibition of Caov-4 cells. Combination of 5 nM dexamethasone with calcitriol caused substantial improvement in growth inhibition (Figure 4A). Dexamethasone at 50 nM caused approximately 40% growth inhibition of Caov-4 cells when administered singly or in combination with calcitriol (Figure 4A), which suggested negligible combined effects at this dexamethasone concentration. As in the experiment with MCF-7 cells, the concentration–response profile of dexamethasone in Caov-4 cells was quite broad and fixed-ratio isobolographic anlaysis was not possible. An isobolographic experiment with constant dexamethasone at 5 nM added to varying calcitriol showed synergistic interations (Figure 4B). Combining 0.5 nM fixed dexamethasone with varying calcitriol resulted in an interaction which bordered between additivity and synergism (Figure 4B).

Discussion

The experiments in this investigation showed that favorable interactions may be obtained for combinations of calcitriol with retinoic acid or dexamethasone in cultured breast and ovarian cancer cells. The synergistic interactions between calcitriol and retinoic acid are consistant with a prior report where the same two agents were examined in T47D breast cancer cells.[13] To our knowledge, this is the first report of synergistic interactions between calcitriol and retinoic acid in ovarian carcinoma cells, and the

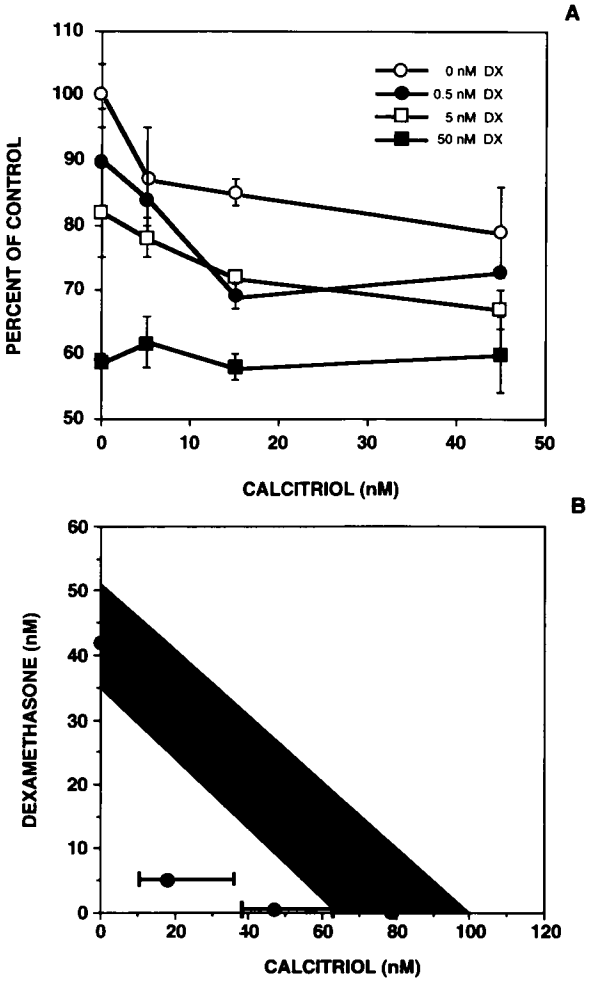

Figure 4. Combined effects of dexamethasone and calcitriol on Caov-4 ovarian carcinoma cell growth. Cells were incubated and growth effects determined as described above. Dexamethasone concentrations (DX) are given in the inset in (A). (A) Means ± SE. (B) Isobologram with constant dexamethasone (0.5 and 5 nM) and varying calcitriol. Dexamethasone–calcitriol interactions were evaluated using IC_{20}s.

first report of synergistic interactions between calcitriol and dexamethasone in breast or ovarian carcinoma cells. The combination of calcitriol and dexamethesone, in MCF-7 breast cancer cells, gave the largest overall effect observed in the investigation, with up to 90% growth inhibition. Isobolographic analyses of these data showed synergistic interactions at the two dexamethasone concentrations evaluated. In Caov-4 ovarian cancer cells the combination of calcitriol and dexamethasone was only able to elicit 42% growth inhibition, even though the two agents acted synergistically with 5 nM dexamethasone. The combination of calcitriol and retinoic acid caused up to 70% growth inhibition of OVCAR3 ovarian cancer cells and acted synergistically at 2:1 and 1:1 ratios of retinoic acid to calcitriol. In OVCAR3 cells greater than 50% growth inhibition was observed with 50 nM calcitriol; however, at this calcitriol concentration retinoic acid provided no additional growth inhibition versus calcitriol alone.

The data in the current study indicate that the ratio at which calcitriol and retinoic acid are combined is highly important, with some ratios resulting in synergism and other ratios giving results that ranged from additivity to non-interaction. It was not possible to determine the ratio-dependence of calcitriol–dexamethasone interactions in this study due to the broad concentration–response profile to dexamethasone. However, the isobologram analyses of calcitriol–dexamethasone combinations did show that the degree of interaction between these two agents was dependent upon dexamethasone concentrations.

The strategy for therapeutic application of cytotoxic anticancer agents is to kill the cancer cells as rapidly as possible, with toxic side effects being the limiting factors for level and duration of drug dose. Conversely, cytostatic agents typically do not kill cancer cells directly, but rather inhibit cell proliferation causing the cells to remain in a non-dividing (G_0) state until they die by natural processes such as senescence or programmed cell death. Cytostatic agents tend to produce fewer and milder side effects and thus may be used for extended courses of therapy. For example, tamoxifen, a cytostatic antiestrogen, is often administered for 2 years and sometimes as long as 5 years.[2] Since calcitriol, retinoic acid and glucocorticoids are naturally occurring 'biologic' agents, these antineoplastics may produce fewer side effects than conventional cytotoxic agents when administered to patients. If, based on studies like the present one, these agents can be given under conditions which create synergistic anticancer effects, the ratio of anticancer response to side effects may be even further improved. In addition, to be therapeutically effective, cytostatic agents must exert strong antiproliferative effects. Clearly, the calcitriol–dexamethasone combination in breast cancer cells (90% inhibition) appears much more promising than the same hormone combination in ovarian cancer cell (42%) inhibition. Next generation analogs of calcitriol, retinoic acid and dexamethasone, in various stages of development and testing, are expected to give improved anticancer activity and even fewer side effects than the analogs used in the present investigation.

Long-term *in vitro* survival experiments and *in vivo* experiments will be necessary to provide greater predictive power of the potential efficacy of such combinations in patients. Such experiments are more involved and are reserved for future investigations. Using the present culture conditions, with longer exposure times and higher calcitriol concentrations, growth arrest of MCF-7 and OVCAR3 cells is observed (data not shown), which suggests that *in vitro* survival experiments may yield favorable results.

The primary tasks accomplished by this investigation were to develop an *in vitro* model in which binary combinations of anticancer agents, hormonal in this case, could be readily evaluated for their degree of interaction against the proliferation of specific types of cancer cells and to use this system to identify conditions which provided optimum interaction between the hormone pairs that were tested. Thus different experimental conditions, such as agent concentrations, drug ratio (see above), exposure time and IC values selected for isobolographic analysis may yield different degrees of drug interaction than observed in the present investigation. The current experiments also provide a framework in which the mechanisms underlying the synergistic hormone interactions could be investigated by virtue of there being a proximal endpoint (growth inhibition at 3 days) which may be compared with cellular effects, such as changes in oncogene expression.

It is possible that the interactions between the drug pairs involves repression of the c-*myc* protooncogene product, a protein that regulates proliferation in breast and other cancers.[23] This laboratory has demonstrated that calcitriol, retinoic acid and dexamethasone all cause reduction of c-*myc* levels in MCF-7 and OVCAR3 cells.[12] Thus is is plausible that the combined drug effects observed in the current experiments could involve joint action of these agents on c-*myc* levels. Further experimentation will be necessary to determine the role of c-*myc* in the growth inhibitory effects of these hormone combinations.

The hormone combinations examined in this study have shown favorable interactions in non-epithelial cells but had until now received little attention in epithelial cancers such as breast and ovarian. Dexamethasone and calcitriol acted synergistically to induce differentiation and inhibit growth of M1 myeloid leukemic cells.[24] These hormones also acted synergistically to induce phagocytosis in the P388D1 macrophage cell line.[25] In rat osteosarcoma cells, the glucocorticoid agonist triamcinolone acetate had no effect when administered separately, but it enhanced the effect of calcitriol in stimulating bone γ-carboxyglutamic acid-containing protein.[26] Calcitriol and retinoic acid synergistically induced phagocytes in P388D1 macrophage cells.[25] In rat osteosarcoma cells, retinoic acid had no effect by itself on γ-carboxyglutamic acid-containing protein synthesis, but signficantly enhanced calcitriol effects on the synthesis of this enzyme.[26] Also in rat osteosarcoma cells, retinoic acid and calcitriol additively reduced parathyroid-induced adenylate cylcase activity.[27]

Conclusion

This study has shown enhancement of growth inhibitory actions ranging from negligible to synergistic when calcitriol was combined with dexamethasone or retinoic acid in breast cancer and ovarian cancer cell lines. The information from this study, degree of interaction, ratio dependence and concentration dependence for the drug interactions may be useful for future, *in vivo*, evaluations of these agents.

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