Anti-Cancer Drugs

CELL CYCLE ARREST AND OXIDATIVE STRESS CONTRIBUTE TO THE ANTIPROLIFERATIVE ACTION OF BSO AND CALCITRIOL ON CACO-2 CELLS
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Dear Editor
Dr Mels Sluyser
Anti-Cancer Drugs

Dear Sirs:

Please find attached the proposal for an Original Research Article entitled "**Cell cycle arrest and oxidative stress contribute to the antiproliferative action of BSO and calcitriol on Caco-2 cells**", by Liaudat Ana C et al., to be considered to publication in your journal. All authors declare that there are no conflicts of interests and this manuscript has not been published or simultaneously submitted for publication elsewhere.

Our work suggests the possibility to alter the sensitivity of colon cancer cells to vitamin D using oxidant drugs. The aim of the present study was to evaluate the effects of BSO, $1,25(OH)_2D_3$ and their combination on intestinal Caco-2 cell growth, in order to elucidate the possible cellular mechanisms involved in their antiproliferative action and to know whether BSO acts as a sensitizer to $1,25(OH)_2D_3$ treatment, which may allow minimizing the toxic effects caused by high doses of the steroid alone.

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Sincerely,

Gabriela Picotto, PhD

CELL CYCLE ARREST AND OXIDATIVE STRESS CONTRIBUTE TO THE ANTIPROLIFERATIVE ACTION OF BSO AND CALCITRIOL ON CACO-2 CELLS

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Running head: Antiproliferative action of BSO and calcitriol on Caco-2 cells

ABSTRACT

Colon cancer prognosis and incidence are connected with vitamin D_3 serum levels. The sensitivity of colon cancer cells to vitamin D could be enhanced by oxidant drugs. Objective: To evaluate the response of colon cancer cell growth to $1,25(OH)_2D_3$ and D,L-buthionine-S,R-sulfoximine (BSO). Methods: Human colon cancer Caco-2 cells were treated with $1,25(OH)_2D_3$, BSO, both or vehicle at different doses and times. Cell proliferation was evaluated by crystal violet staining. Cell cycle and mitochondrial membrane potential were measured by flow cytometry. Total glutathione levels, catalase, superoxide dismutase and alkaline phosphatase activities were analyzed by spectrophotometry. DNA fragmentation was evaluated by TUNEL assay. Results were statistically analysed by one way ANOVA and Bonferroni as a post-hoc test. Results: BSO and $1,25(OH)_2D_3$ inhibited Caco-2 cell growth, effect that was higher with the combined treatment. BSO *plus* $1,25(OH)_2D_3$ induced cell cycle arrest and suppressed cell division. Total glutathione levels decreased either with BSO or BSO *plus* $1,25(OH)_2D_3$. Catalase activity increased with the combined treatment. Mitochondrial membrane potential and alkaline phosphatase activity were altered by $1,25(OH)_2D_3$ alone or *plus* BSO. Percentage of TUNEL positive cells was increased. Conclusion: BSO increases the antiproliferative effect of $1,25(OH)_2D_3$ on Caco-2 cells through oxidative stress induction that causes DNA breakage. The antioxidant system can partially compensate the damage induced by BSO *plus* $1,25(OH)_2D_3$. The increment observed in alkaline phosphatase activity suggests that cell differentiation induction also mediates the effect of the combined treatment.

Keywords: $1,25(OH)_2D_3$, D,L-buthionine-S,R-sulfoximine, Caco-2 cells, oxidative stress, cell cycle and differentiation.

Abbreviations: BSO, DL-buthionine-S,R-sulfoximine; MEN, menadione; GSH, glutathione; 25(OH)D$_3$, 25 hydroxyvitamin D$_3$; D, 1,25(OH)$_2$D$_3$; ROS, reactive oxygen species; MEM, Minimum Essential Media; PBS, phosphate buffered saline; FBS, fetal bovine serum; PMSF, phenyl methanesulphonyl fluoride; PI, propidium iodide; DAPI, 4,6-diamidino-2-phenylindole; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; $\Delta\Psi$m, mitochondrial membrane potential; DiOC$_6$, 3,3´-dihexyloxacarbocyanine iodide; AP, alkaline phosphatase; TUNEL, terminal dUTP nick end labeling.

INTRODUCTION

Colorectal cancer is one of the leading causes of tumor death in the entire world and its chemotherapeutic treatment is always a subject of intense study [1]. The incidence and prognosis of this disease are suggested to be closely related to plasma $25(OH)D_3$ (calcidiol) levels, an indicator of nutritional status of vitamin D, although $1,25(OH)_2D_3$ or calcitriol is the most active biological molecule of vitamin D_3. Epidemiological studies suggest that high concentrations of calcidiol diminish the risk of colon cancer. It has been estimated that an increment of 25 nmol/L in serum $25(OH)D_3$ levels (normal values: 75 nmol/L or 30 ng/mL) [2] reduces the incidence of the disease by 17% and mortality by 29% [3]. A metaanalysis showed that increases of 20 ng/mL in serum $25(OH)D_3$ levels reduced the risk of colon and rectal cancer in 59% and 22% of cases, respectively [4].

The best known role of $1,25(OH)_2D_3$ is the regulation of calcium and phosphorus homeostasis, favoring intestinal Ca^{2+} absorption [5]. Numerous non classical actions of vitamin D_3 have been described, including the modulation of cell growth and adhesion, cell cycle arrest, induction of differentiation, stimulation of apoptosis, inhibition of angiogenesis, among others [6, 7]. Decreased cell growth by the secosteroid was demonstrated in both benign (e.g. psoriasis) and malignant cells derived from different tissues such as breast, pancreas, intestine, prostate and skin [8-14].

On the other hand, many studies showed that the resistance observed in different tumors is usually associated with overexpression of antioxidant molecules in malignant cells, being this an important cause of failure of anticancer therapy [15]. High levels of glutathione (GSH) are in part responsible for this resistance [16]. GSH is the main intracellular tripeptide that regulates redox balance and is involved in several processes

as the regulation of cell proliferation or death mechanisms such as apoptosis, necrosis [17] and autophagy [18]. When the oxidative stress occurs, the most important organelles targeted for damage are the nucleus and mitochondria. The exacerbation of reactive oxygen species (ROS) can induce injury of cell structures, altering molecules as lipids, proteins and nucleic acids [19, 20]. Therefore, the use of compounds that stimulate oxidative stress such as D,L-buthionine-S,R-sulfoximine (BSO), menadione (MEN) and azathioprine, may increase the sensitivity of malignant cells to conventional chemotherapy. BSO is a synthetic amino acid that irreversibly inhibits the activity of γ-glutamylcysteine synthetase, limiting step enzyme in GSH synthesis [21]. Due to the tumor resistance is associated with high intracellular GSH levels, BSO is used in clinical practice [15, 22]. In our laboratory we have demonstrated that the combination of BSO *plus* $1,25(OH)_2D_3$ enhances the antiproliferative effect on breast cancer MCF-7 cells produced by each single drug [8]. We have also shown that the combined treatment induces apoptosis via oxidative stress, as occurs with the exposure of MCF-7 cells to $1,25(OH)_2D_3$ *plus* MEN [10]. Pretreatment of the human hepatocellular carcinoma derived HepG2 cells with BSO increased cellular cytotoxicity induced by salicylic acid [23]. Similarly, the combined treatment of BSO *plus* 17-β estradiol enhanced cell death by apoptosis either of mice xenografts or MCF-7 cells resistant to estrogen therapy [16].

The aim of the present work was to evaluate the effects of BSO, $1,25(OH)_2D_3$ and their combination on intestinal Caco-2 cell growth, in order to elucidate the possible cellular mechanisms involved in their antiproliferative action and to know whether BSO acts as a sensitizer to $1,25(OH)_2D_3$ treatment, which may allow minimizing the toxic effects caused by high doses of the steroid alone. Our data indicate that BSO increases the antiproliferative effect of $1,25(OH)_2D_3$ on Caco-2 cells through oxidative stress

induction. The increment observed in alkaline phosphatase activity suggests that cell differentiation induction also mediates the effect of the combined treatment.

MATERIAL AND METHODS

Chemicals. Minimum Essential Media (MEM) and phosphate buffered saline (PBS) were purchased from Gibco (Invitrogen, Grand Island, NY, USA). Fetal bovine serum (FBS) was from Natocor (Carlos Paz, Córdoba, Argentina). DL-buthionine-S,R-sulfoximine (BSO) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS according to the manufacturer's instructions. $1,25(OH)_2D_3$ was a generous gift from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). All other reagents were of analytical grade.

Cells and cell culture. Human colon carcinoma Caco-2 cell line was maintained in MEM supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 ºC in an atmosphere of 95% air 5% CO_2 and was subcultured (0.25% Trypsin-EDTA, Sigma-Aldrich) once a week. Cells were plated and grown in a complete media for 24 h and then treated with BSO in the presence or absence of $1,25(OH)_2D_3$ for different times at the indicated concentrations. Cells treated with 0.05 % ethanol vehicle were included as a control group in all assays.

Cell growth assay and morphology. Adherent cell number was evaluated as previously described [24]. Briefly, cells seeded at 6 x 10^3 cells/well in 24-well plates were allowed to attach for 24 h and then exposed to BSO, with or without $1,25(OH)_2D_3$, or ethanol at the indicated times. Cells were then fixed with 1% glutaraldehyde, incubated with 0.1% crystal violet, destained with H_2O and solubilized with 0.2% Triton X-100. Absorbance, which is proportional to adherent cell number under the conditions used, was measured at 570 nm. Cell morphology was assessed by phase contrast using a Leica inverted microscope (Leica Microscopy and Scientific Instruments Group, Buffalo, NY, USA).

Cell cycle analysis. Cells were harvested by trypsinization, collected by centrifugation, fixed in 100% ethanol (-20°C) and incubated with 5 µg/mL propidium iodide (PI) and 0.015 U/mL RNase in PBS for 20 min at room temperature, as previously described [8]. Cell cycle analysis was performed using a BD FACSCanto[TM]II flow cytometer (BD Biosciences, San Jose, CA, USA). PI was analyzed employing a 564–606 nm band pass filter. Data were modeled using the FACSDiva software (BD Biosciences).

Cell mitotic nuclei. Cells were trypsinized and seeded (3 x 10^5 cells/well) on glass slides from six-chamber over glass slides for 24 h and then treated with BSO and/or $1,25(OH)_2D_3$. The slides were rinsed with PBS, fixed in 100% methanol overnight, permeabilized 10 min with 0.1 % Triton X-100 and finally incubated for 30 min with 1 µg/mL 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at room temperature. Image acquisition of DAPI staining (filter range: excitation 360 ± 40 nm and fluorescence emission 460 ± 50 nm) was performed using a fluorescent microscope Leica Micro Star IV (Leica Microscopy and Scientific Instruments Group) [25]. The percentage of mitotic cells represents the number of mitotic nuclei in relation to the total number of nuclei.

Preparation of cell extracts. Cell homogenates were employed to assay the total GSH levels and the activity of superoxide dismutase (SOD) and catalase (CAT) antioxidant enzymes and alkaline phosphatase (AP). After treatments, cells were rinsed twice with PBS, resuspended and homogenized in lysis buffer (1 mM PMSF, 1 mM NaF and 1% Triton X-100). Lysates were centrifuged for 15 min at 10,000 rpm and the supernatants were stored at -20ºC until the experiments. Protein content from homogenates was determined using purified serum albumin as standard [26].

Total GSH determination. Total GSH levels were measured using the glutathione disulfide reductase-5,5'-dithiobis (2-nitrobenzoate) recycling procedure, as described elsewhere [27].

Superoxide dismutase and catalase activities. SOD activity was determined in 1 μM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 40 μM riboflavin, 50 mM potassium phosphate buffer (pH 7.8). Reduction of NBT was measured spectrophotometrically at 560 nm, as previously described [28]. CAT activity was assayed in 50 mM potassium phosphate buffer (pH 7.4) and 0.3 M H_2O_2. The H_2O_2 decomposition rate was monitored at 240 nm and was directly proportional to enzyme activity [29].

Mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\Psi m$) was assessed by cell staining with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$, Invitrogen). After treatments, cells (2×10^5) were resuspended in 500 μL of PBS containing 40 nM $DiOC_6$ and incubated in darkness for 15 min at 37ºC. Fluorescence intensity of $DiOC_6$ was analyzed in a BD FACSCanto^{TM}II flow cytometer with excitation and emission settings of 484 and 500 nm, respectively and the results analyzed with the FACSDiva software [30].

TUNEL assay. Detection of DNA strand breaks was performed with the *in situ* cell death detection kit (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions. Cells were grown, fixed with paraformaldehyde (3%) at 4ºC and permeabilized with ethanol/acetic acid solution (2:1) during 15 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 5 min. Images were obtained with a Leica Micro Star IV microscope. The percentage of nuclear DNA fragmentation was expressed as the number of TUNEL positive cells in relation to the total cell number.

Alkaline phosphatase activity assay: AP activity was determined employing 3 mmol/L p-nitrophenyl phosphate as a substrate in 0.5 mol/L diethanolamine buffer (pH 9.8). The mixture was incubated at 37 ºC for 30 min and the reaction was stopped with 1 M NaOH. Absorbance was measured at 405 nm [31].

Statistical analysis: Data were evaluated by one-way analysis of variance (ANOVA) and Bonferroni *post hoc* test using SPSS software for Windows Release 20.0.0 Standard Version (IBM Corporation, Armonk, New York, USA). Differences were considered significant at $p < 0.05$.

RESULTS

BSO and 1,25(OH)$_2$D$_3$ decreased Caco-2 cell growth

Different doses of both BSO and 1,25(OH)$_2$D$_3$ were tested on the Caco-2 intestinal cell line in order to evaluate the effects of the compounds on cell growth. Cell proliferation decreased in a dose-dependent fashion (not shown) and resulted maximal when cells were treated with 100 μM BSO and 200 nM 1,25(OH)$_2$D$_3$ for 96 h (70 and 19% of inhibition compare to the control value, respectively) (Fig. 1A). BSO *plus* 1,25(OH)$_2$D$_3$ together decreased cell growth by 82 % at 96 h (**p <0.05 *vs.* BSO; *vs.* 1,25(OH)$_2$D$_3$) which resulted even higher than that obtained by using individual treatments with BSO or 1,25(OH)$_2$D$_3$ (Fig. 1A). Cell growth did not change at early exposure time (6 h) with any of the treatments. However, the inhibition of proliferation was significant when cells were exposed to BSO or the combined treatment after 48 h and no effect of 1,25(OH)$_2$D$_3$ alone was obtained. Caco-2 cell morphology under BSO, 1,25(OH)$_2$D$_3$ or the combined treatment was evaluated by phase contrast microscopy. Cell exposure to vehicle (control) showed a typical epithelial morphology and high density. However, when single drugs were used, the cell confluence decreased being this effect more remarkable with the combined treatment. The cells showed shrunk size and altered cellular morphology under the different treatments (Fig. 1B).

Cell cycle was altered by BSO *plus* 1,25(OH)$_2$D$_3$

We analyzed whether the changes in cell growth patterns induced by the compounds could be a consequence of cell cycle alteration. Percentages of treated and non treated cells in G$_0$/G$_1$ and S+G$_2$/M phases were obtained by flow cytometry. As depicted in Table 1, samples treated with BSO *plus* 1,25(OH)$_2$D$_3$ for 48 h presented an increase in distribution of cells in S+G$_2$/M phase (45 % above control value).

Consequently, there was a decreased percentage of cells in G_0/G_1 phase, suggesting a delayed S to mitosis transition. The individual administration of BSO or $1,25(OH)_2D_3$ did not alter cell cycle progression. Moreover, to evaluate if $S+G_2/M$ arrest induced by BSO *plus* $1,25(OH)_2D_3$ could be related to changes in mitosis, the percentage of cells in division process was analyzed by DAPI staining. Only Caco-2 cells treated with BSO *plus* $1,25(OH)_2D_3$ during 96 h showed a significant decreased percentage of cells in mitosis phase (Fig. 2).

BSO and its combination with $1,25(OH)_2D_3$ changed the cellular antioxidant system

In order to identify whether the antioxidant system was altered by the different treatments, we evaluated the total GSH levels and the activity of CAT and SOD. Caco-2 cells were incubated with 100 μM BSO, 200 nM $1,25(OH)_2D_3$ and their combination at different times. Intracellular total GSH concentration was decreased in cells treated with BSO and BSO *plus* $1,25(OH)_2D_3$ at 6 and 48 h while no differences between groups were observed after 96 h (Fig. 3). SOD activity did not change with the different treatments at any time tested. CAT activity showed no alteration, at 6 and 48 h (data not shown), but increased after 96 h of BSO *plus* $1,25(OH)_2D_3$ treatment (Fig. 4).

BSO and $1,25(OH)_2D_3$ altered the mitochondrial membrane potential

Alterations in cellular redox state may lead to changes in mitochondrial membrane potential. Caco-2 cells were treated with 200 nM $1,25(OH)_2D_3$ and/or 100 μM BSO for 96 h and $\Delta\psi m$ was analyzed by $DiOC_6$ staining. The percentage of cells presenting lower mitochondrial membrane potential increased when cells were treated with $1,25(OH)_2D_3$ or BSO *plus* $1,25(OH)_2D_3$, being the latter more pronounced (Fig. 5).

Although no effects of BSO on $\Delta\psi$m were observed at 96 h, an increase in the percentage of Caco-2 cells with low $\Delta\psi$m was produced at 48 h similar to that observed with $1,25(OH)_2D_3$ or BSO *plus* $1,25(OH)_2D_3$ (69.7%, 70.6% and 72.1% of control value for BSO, $1,25(OH)_2D_3$ and BSO *plus* $1,25(OH)_2D_3$ respectively).

BSO and $1,25(OH)_2D_3$ induced DNA fragmentation

To evaluate if changes in redox state produce alterations in DNA structure, we tested the presence of DNA fragmentation by detection of free 3´ hydroxyl ends of the fragmented DNA. The percentage of TUNEL positive cells increased when they were exposed to BSO ($4.99* \pm 0.87\%$) and $1,25(OH)_2D_3$ ($4.35* \pm 0.21\%$) for 96 h compared to control values ($0.57 \pm 0.29\%$). The effect of BSO *plus* $1,25(OH)_2D_3$ on DNA fragmentation was more pronounced ($20.34** \pm 0.59\%$) than that individual treatments ($*p<0.05$ *vs* control, $**p<0.05$ *vs* BSO and $1,25(OH)_2D_3$).

$1,25(OH)_2D_3$ enhanced Caco-2 cell differentiation

Figure 6 shows that AP activity, a cell differentiation marker enzyme, did not change when cells were treated with 100 µM BSO and/or 200 nM $1,25(OH)_2D_3$ for 6 or 48 h. However, the enzyme activity increased when cells were exposed to $1,25(OH)_2D_3$ or BSO *plus* $1,25(OH)_2D_3$ for 96h. The response to the combination of both drugs resulted not different to that from $1,25(OH)_2D_3$ single treatment.

DISCUSSION

The present work demonstrates that BSO enhances Caco-2 sensitivity to $1,25(OH)_2D_3$ producing a reduction of cell proliferation via cell cycle arrest and oxidative stress. Concomitant exposure of cell cultures to BSO and the steroid hormone highly increases the inhibition of cellular proliferation over the individual effects of each drug and results dependent on the concentration and time of exposure to the different treatments. The remarkable inhibitory effect of BSO *plus* $1,25(OH)_2D_3$ is elicited at the fourth day of treatment and is produced, at least in part, by cell cycle arrest in $S+G_2/M$ phase and reduction of mitotic division. The antioxidant system is also modified as judged by the increment in CAT activity and alteration of the mitochondrial membrane potential. The combined drugs produce GSH depletion at earlier times of exposure (6 and 48 h) and DNA fragmentation at 96 h. A rise in AP activity indicates that cell differentiation is enhanced by BSO *plus* $1,25(OH)_2D_3$. The present data clearly indicate that the molecular events triggered by BSO, $1,25(OH)_2D_3$ and the combined treatment occur at different times of exposure.

Our results show that $1,25(OH)_2D_3$ alone reduces Caco-2 cell proliferation at about 20%. These results confirm the inhibitory effect of $1,25(OH)_2D_3$ on this colon cancer cell line, as previously observed [32]. However, the antiproliferative action of $1,25(OH)_2D_3$ on Caco-2 cells is less pronounced than that shown in other epithelial cells, such as the breast cancer MCF-7 cells. We have demonstrated that $1,25(OH)_2D_3$ decreases MCF-7 cell growth in 50 % approximately [8]. This suggests that Caco-2 cells may be more resistant to the secosteroid action. Although it is well known that $1,25(OH)_2D_3$ mainly affects calcium and phosphorous homeostasis, the inhibition of neoplastic cell growth is considered as a "nonclassical" effect of $1,25(OH)_2D_3$ [33]. In this line, it has been reported that $1,25(OH)_2D_3$ decreases cell proliferation on breast,

colon and prostate cancer cells as well as on tumor endothelial cells and T cell regulatory system [6, 8, 10, 34-38]. In addition, Caco-2 cells show that $1,25(OH)_2D_3$ alters mitochondrial membrane potential, which may be relevant to stimulate oxidative stress although the steroid alone does not produce GSH depletion or modifications in the antioxidant enzyme activities. DNA fragmentation (TUNEL positive cells) is enhanced by $1,25(OH)_2D_3$ treatment. It is known that calcitriol is involved in cell differentiation [34] and, as expected, $1,25(OH)_2D_3$ increases AP activity.

BSO, a GSH synthesis inhibitor, has been tested in clinical trials for anticancer treatment to enhance the cytotoxicity of conventional therapies [39]. In our study we have demonstrated that the antiproliferative effect produced by BSO on Caco-2 cells is accompanied by an increase in oxidative stress and DNA fragmentation. The latter possibly could be a consequence of the antioxidant system alteration. GSH decreases at short times of exposure (6 and 48 h) and returns to control levels at 96 h. Moreover, depletion of total tripeptide levels is related to low mitochondrial membrane potential, both indicative events of oxidative stress.

The tripeptide GSH and the enzymes SOD and CAT constitute the most important molecules representative of the antioxidant defense system [19]. Oxidative stress produces harmful effects on mitochondrial function and DNA integrity [40]. In this study, we observed that the combination treatment decreases GSH levels at earlier times while CAT activity increases much later. These results suggest that GSH is the primary antioxidant defense to low continuous oxidants and CAT become more important when redox status is severely modified [19]. The alteration of these parameters, in association with disruption of mitochondrial membrane potential, reinforces the idea that the sensitizing effect observed with the BSO *plus* $1,25(OH)_2D_3$ treatment is due, in part, to an alteration of the cellular redox state.

Cell cycle is a process regulated by growth factors that control different cellular pathways such as proliferation [41]. Our work constitutes the first evidence of cooperative effect of BSO with $1,25(OH)_2D_3$ to produce Caco-2 cell cycle arrest in S+G2/M phase. However, at the studied doses each compound does not cause changes in the cell cycle distribution. Other authors demonstrate that BSO [17] and $1,25(OH)_2D_3$ [42] single treatments alter colon cancer cell cycle progression but the doses employed were much higher than those we have used. In addition, have been found no alterations in cell cycle in Caco-2 cells treated with $1,25(OH)_2D_3$ for 3-4 days [12]. Nonetheless, the combination treatment decreases mitotic division and induces cell differentiation, as indicated by the enhancement of AP activity [43]. Cell differentation may be related to antioxidant system alteration induced by combined treatment. Other oxidants (e.g. H_2O_2) produce changes in the glycosylation of AP and, as a consequence, an increase in the enzyme activity of different intestinal cell lines [44]. The importance of stimulating cell differentiation is related to proteomic changes induced by this state. The expression of proteins associated with proliferation, cell growth and cancer evolution are downregulated upon cellular differentiation, promoting a cancer phenotype less aggressive than undifferenciated cells [45].

BSO *plus* $1,25(OH)_2D_3$ produce cellular morphology alteration and DNA fragmentation. Other investigators [46] have demonstrated that oxidative stress causes changes in membrane integrity and significant DNA damage, which is in agreement with our results and support the increased susceptibility induced by the combined treatment on Caco-2 cells. Further studies are going on to identify which cell death program is activated by BSO *plus* $1,25(OH)_2D_3$.

In conclusion, oxidative stress and cellular differentiation induced by BSO *plus* $1,25(OH)_2D_3$ appear to be a suitable combination to potentiate the sensitivity of Caco-2

cells to death. BSO increases the antiproliferative effect of $1,25(OH)_2D_3$ on Caco-2 cells, an effect triggered by oxidative stress and cell cycle arrest, leading finally to cell differentiation and cell death. *In vivo* studies are needed to test whether the combined action of BSO *plus* $1,25(OH)_2D_3$ may be useful as an alternative therapy for colon cancers that became resistant to other conventional treatments and possibly attenuate the hypercalcemic effects evoked by calcitriol at doses required to reduce tumor growth [47].

ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Fig. 1: BSO and 1,25(OH)₂D₃ (D) decreased Caco-2 cell growth and modified cell morphology. Cells were treated with ethanol, 100 µM BSO, 200 nM D or BSO *plus* D during the indicated times. A) Cell growth was evaluated by cristal violet staining. The values represent optical density (OD) means ± S.E. *p<0.05 *vs* control, **p<0.05 *vs* BSO or D. B) Phase contrast images (magnification 400x) of Caco-2 cells treated as indicated above during 96 h. Arrows indicate alterations in cellular morphology.

Fig. 2: Effect of BSO and 1,25(OH)₂D₃ (D) on mitotic division of Caco-2 cell. Cells were treated with 100 µM BSO, 200 nM D and BSO plus D for 96 hours. The values represent the percentage of cells in mitotic state. Values means ± S.E. *p<0.05 *vs.* Control.

Fig. 3: BSO and BSO *plus* 1,25(OH)₂D₃ (D) depleted GSH levels in Caco-2 cells. Cells were treated with ethanol, 100µM BSO and/or 200 nM D during 6, 48 and 96 h. Total GSH levels were measured by spectrophotometry, as indicated under methods. The values represent means ± S.E from three independent experiments performed in triplicate.*p<0.05 *vs* control.

Fig. 4: BSO *plus* 1,25(OH)₂D₃ (D) increased catalase activity. Caco-2 cells were treated with ethanol, 100 µM BSO, 200 nM D or BSO *plus* D for 96 h. CAT activity was analyzed, as indicated under methods. Values are means ± S.E from at least three independent experiments performed in triplicate. *p< 0.05 *vs* control.

Fig. 5: Alterations induced by BSO and 1,25(OH)$_2$D$_3$ (D) on mitochondrial membrane potential in Caco-2 cells. Cells were treated with ethanol, 100 µM BSO, 200 nM D or BSO *plus* D for 96 h, stained with DiOC$_6$ and ($\Delta\Psi$m) analyzed by flow cytometry, as described in methods. The histograms are representative of three independent experiments.

Fig. 6: Changes in alkaline phosphatase (AP) activity. Caco-2 cells were treated with ethanol, 100 µM BSO, 200 nM 1,25(OH)$_2$D$_3$ (D) or BSO *plus* D for 6, 48 and 96 h and AP activity was evaluated, as indicated under methods. Values are means ± S.E from at least three independent experiments performed in triplicate. *p<0.05 *vs* control or BSO.

Figure 1

Fig. 1

A

B
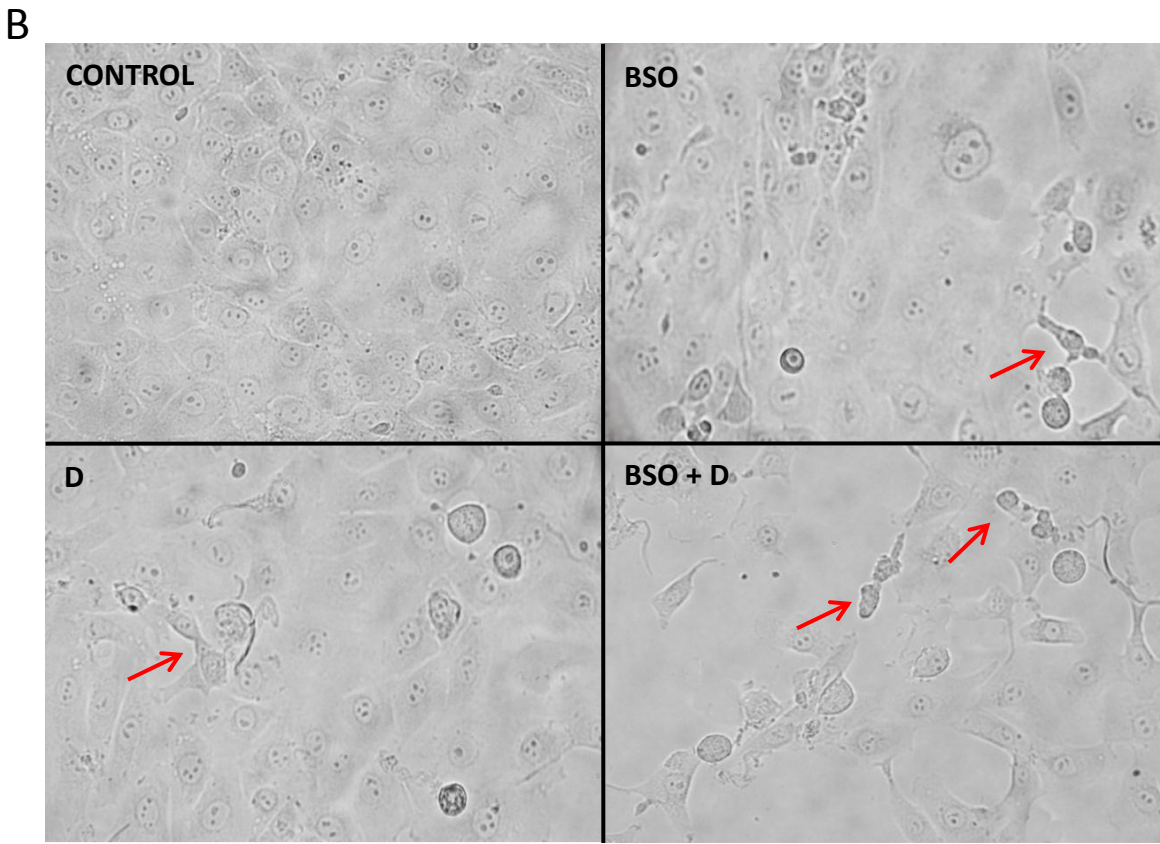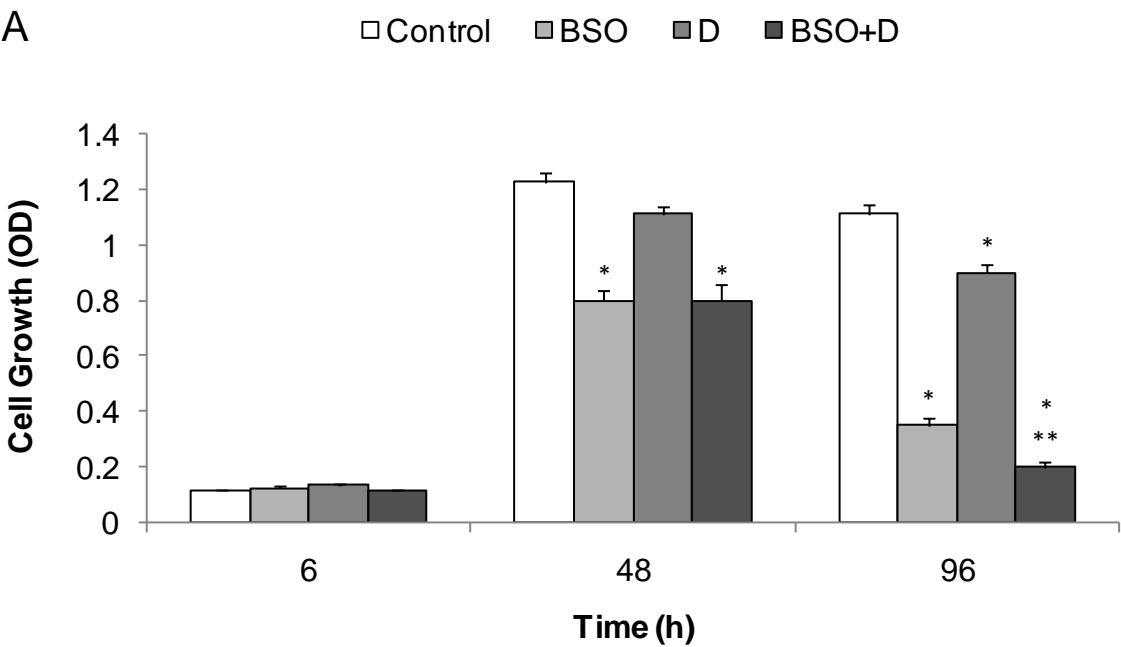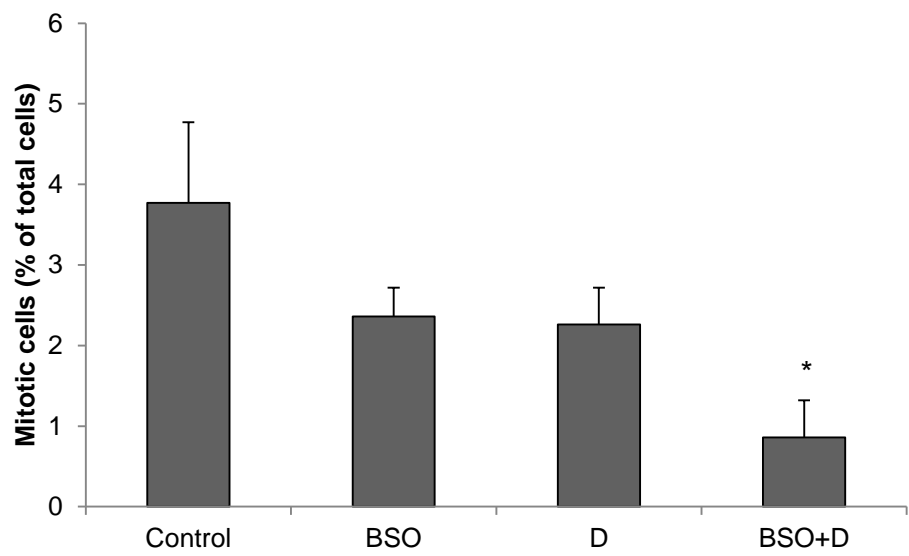
Figure 2

Fig. 2

Figure 3
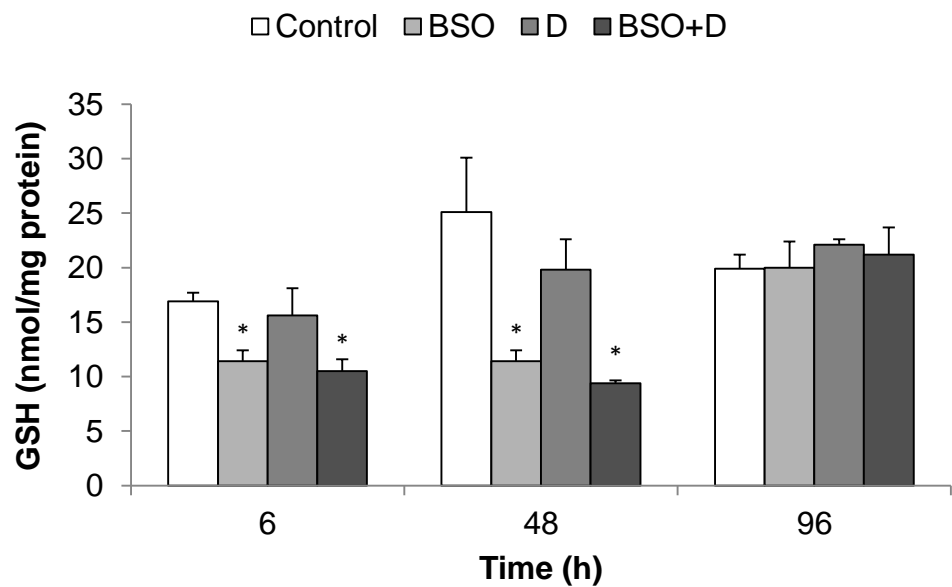
Fig. 3

Figure 4
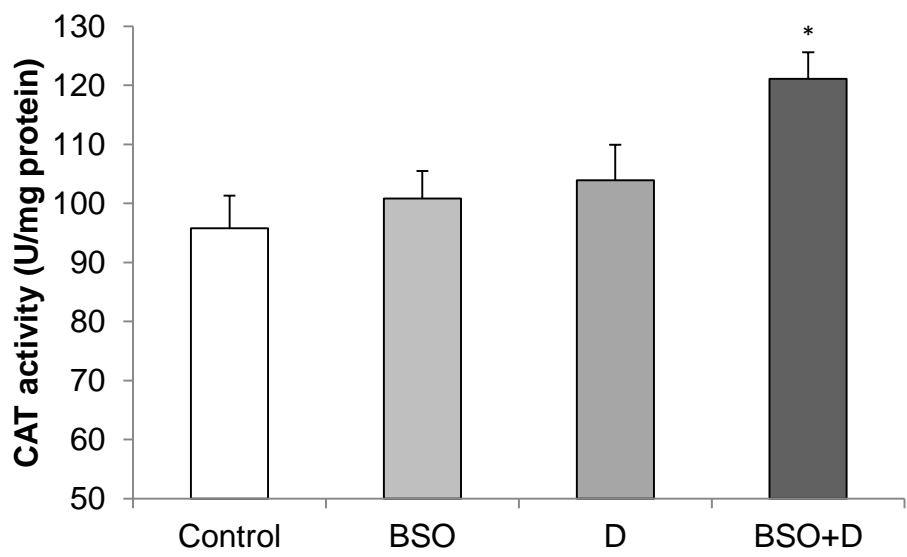
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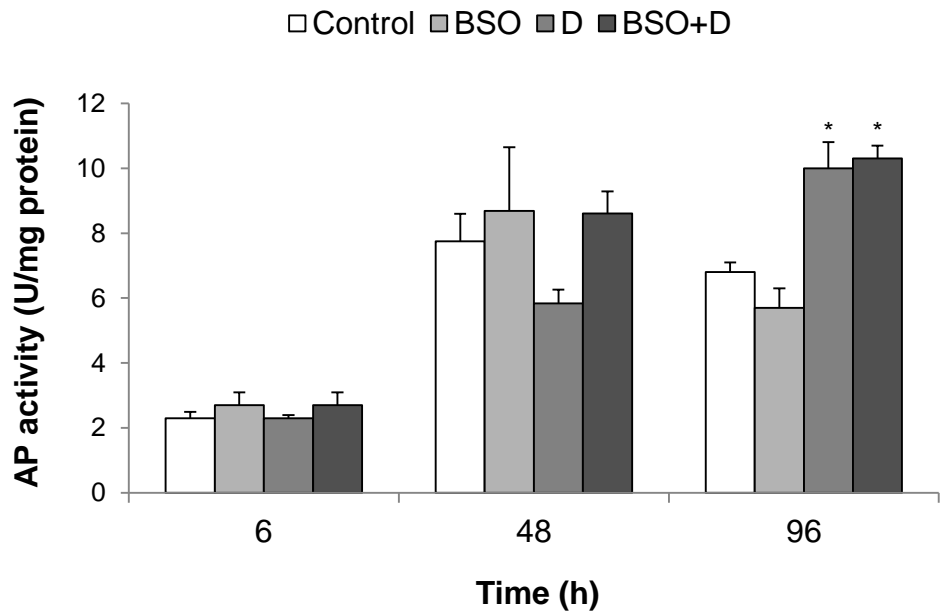
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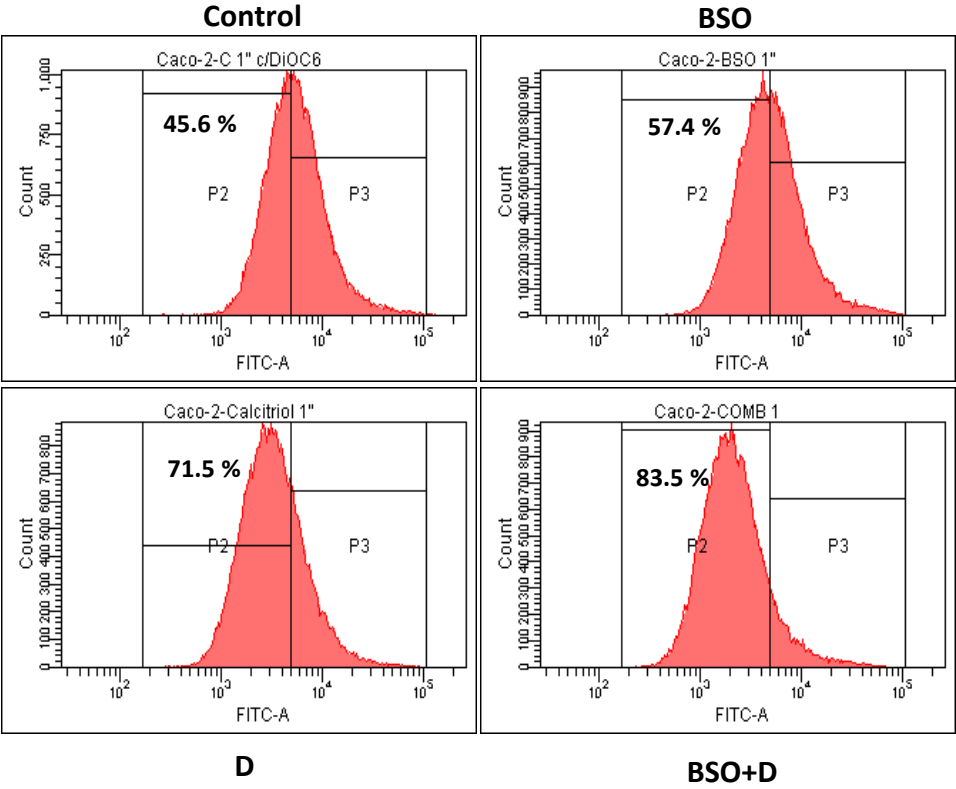
Fig. 5

Figure 6

Fig. 6

Table 1

Table 1. BSO *plus* $1,25(OH)_2D_3$ altered cell cycle of Caco-2 cells.

Treatment	G_0/G_1 (%)	$S+G_2/M$ (%)
Control	77.7 ± 2.3	22.6 ± 2.4
BSO	72.3 ± 3.2	27.4 ± 3.2
D	71.8 ± 0.8	28.0 ± 0.8
BSO+D	$66.8 \pm 0.7^*$	$32.9 \pm 0.8^*$

Caco-2 cells were treated with ethanol (vehicle), 100 µM BSO, 200 nM D or BSO *plus* D for 48 h and cell cycle was analyzed by flow cytometry. Results represent the percentage of control and treated cells in G_0/G_1 and $S+G_2/M$ phases of cell cycle. Values indicate means ± S.E from at least three independent experiments. p* < 0.05 *vs.* each corresponding control.

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