Calcitriol Reverses Induced Expression of Efflux Proteins and Potentiates Cytotoxic Activity of Gemcitabine in Capan-2 Pancreatic Cancer Cells

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ABSTRACT- Purpose. Efflux and influx proteins play a major role in chemo-resistance by affecting the net cellular uptake of anti-cancer drugs. Hence, alteration of the efflux and influx protein expression may result in variations of chemotherapeutics uptake and consequently cell death rate. The present study investigated the effects of pre-treatment of capan-2 pancreatic cancer cells with calcitriol, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) or silibinin on the induction of three major efflux proteins and the main gemcitabine influx protein. The influence of the pre-treatments on the net cellular uptake of gemcitabine, total ATPase activity, and cell death rate were also evaluated. **Methods.** Capan-2 pancreatic cancer cells were pre-treated for 24 h with calcitriol, BHT, BHA, or silibinin, followed by gemcitabine treatment. The concentration of gemcitabine was quantified using ultra-performance liquid chromatography (UPLC). Real-time polymerase chain reaction (RT-PCR) was utilized in order to investigate the expression of the mRNAs. The expression of the proteins was assessed using western blotting. Measurement of the ATPase activity was conducted utilizing a colorimetric method and viability of the cells was determined using a luminescent cell viability assay. **Results.** Protein expression studies showed that BHT, silibinin, and BHA increased expression of the efflux proteins and decreased the overall uptake of gemcitabine, whereas calcitriol significantly inhibited expression of the efflux proteins and increased gemcitabine uptake. Expression of specific mRNAs correlated reasonably well with the levels of corresponding proteins. Additionally, the expression of efflux proteins and ATPase activity were well correlated, signifying that the induced efflux proteins are functionally active. Moreover, pre-treatment with calcitriol resulted in a significant increase in cell death with gemcitabine treatment, whereas, BHA significantly reduced the cell death rate. On the other hand, pre-treatment with BHT and silibinin had no significant effect on the cell death rate. **Conclusions.** Pre-treatment of the pancreatic cancer cells with calcitriol significantly increased gemcitabine cellular uptake and consequently decreased cell viability after treatment with gemcitabine, whereas BHA significantly reduced gemcitabine uptake and decreased cell death rate, which were at least partially attributed to the alteration of expression of efflux and influx proteins.

INTRODUCTION

Despite many advances in molecular and targeted anticancer therapeutics over the past 30 years, pancreatic cancer has remained one of the most aggressive types of malignancies with less than a 5% survival rate within 5 years of diagnosis (1-4). Gemcitabine is considered the "gold" standard chemotherapeutic agent for treatment of pancreatic cancer; however, the response rate to gemcitabine alone or in combination with other chemotherapeutics is only marginal (1, 2, 4, 5). Among many reasons that have been investigated to explain this chemotherapeutic inefficiency (6-11), reduced cellular uptake of gemcitabine as a result of down regulation of influx proteins and/or upregulation of the efflux proteins has been highlighted in several studies (12-15) . The alteration of influx and efflux protein expression may lead to a significant reduction in the intracellular concentration of chemotherapeutic agents, which

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ultimately results in poor response to treatment and development of multiple drug resistance (MDR) against several structurally unrelated anticancer agents (13-18). Gemcitabine is taken up into the cells by various concentrative nucleoside transporter (CNT) and equilibrative nucleoside transporter (ENT) isoforms (19-21). In human pancreatic adenocarcinoma cells including capan-2 cells, the expression of members of the CNT family is negligible (19-23). Therefore, gemcitabine is mostly taken up via the hENT1 transporter, a member of the equilibrative nucleoside transporter efflux proteins family, which is abundantly expressed.

We have shown in our previous investigation that expression of hENT1 transporters is significantly altered during development of gemcitabine resistance (24). Additionally, it has been reported that the sensitivity of pancreatic cancer cell lines to gemcitabine is significantly influenced by MRP5 (ABCC5) efflux protein (20, 24) and to a lesser extent by MRP1 (ABCC1) (25) and P-gp (ABCB1) (25, 26). Therefore, chemicals that can modulate expression of influx and efflux proteins have the potential to substantially alter therapeutic outcomes of chemotherapy.

Synthetic phenolic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are widely used in medicine and food industry and are part of the daily diet, especially in fat containing foods to protect them against oxidation (27, 28). Moreover, it is often the public perception that any additional intake of antioxidants reduces the risk of oxidative stress-related diseases (29) and prevents free radical-induced tissue damage; hence these compounds can assist in coping with oxidative stress conditions (27, 30). In addition to food, BHT is utilized as an antioxidant in other products such as cosmetics and pharmaceuticals and BHA is employed both as a preservative in food products and in manufacturing of some medications (27, 28, 31). Acceptable daily intake of BHA and BHT has been suggested to be 0.5 mg/kg body weight (32, 33). Carcinogenicity of both compounds has been evaluated in animals, in a report by the International Agency for Research on Cancer, in which it has been reported that there is limited evidence for carcinogenicity of BHT in experimental animals and no data are available for the same in humans (34). On the other hand, this report found sufficient evidence for carcinogenicity of BHA in animals, yet still no information is available in humans (34).

Silibinin is the major flavonoid of silymarin, a standardized extract of the milk thistle seeds that conventionally has been used for treatment of liver diseases. Recently, there were reports that this compound caused apoptosis and cell cycle arrest in human pancreatic cancer cells (35) and had an anti-neoplastic effect in other type of cancers (36). It has also been reported that silibinin can inhibit P-glycoprotein (P-gp) mediated efflux (37). The inhibition of P-gp activity may lead to alteration of cellular uptake of drugs that are P-gp substrates.

Calcitriol (1, 25-dihydroxycholecalciferol) is the active metabolite of vitamin D, which plays an important role in regulation of intestinal calcium absorption, and metabolism of bone and minerals (38, 39). It is known that calcitriol has anti-proliferative activity through different mechanisms such as cell cycle arrest and apoptosis (38-41). Additionally, calcitriol can enhance anti-cancer activity of anti-cancer drugs including gemcitabine (42) and paclitaxel (43) both *in vitro* and *in vivo*.

To the best of our knowledge, there has been no study to date on the effect of calcitriol, BHT, BHA or silibinin on the expression of efflux and influx proteins in capan-2 pancreatic cancer cells. Therefore, the objectives of this study were to investigate the modulatory effect of calcitriol, BHT, BHA and silibinin on several efflux/influx proteins and to explore the impact of such modulations on intracellular concentrations of gemcitabine. As any alteration of the efflux and influx protein expression, may result in variations of gemcitabine uptake and consequently cell death rate, we hypothesized that calcitriol, BHT, BHA or silibinin may alter the expression of the efflux/influx proteins, and hence the cellular uptake and removal of gemcitabine will be affected. Therefore, pre-treatment with these compounds, can significantly change the gemcitabine cytotoxicity.

MATERIAL AND METHODS

Materials

BHT, BHA, silibinin, and cellytic M were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcitriol (1, 25-Dihydroxy vitamin D3) was obtained from Tocris Bioscience (Bristol, United Kingdom). Gemcitabine was acquired from Biotang (Lexington, MA, USA). The capan-2 cell line, McCoy's 5A medium, and fetal bovine serum were acquired from American Type Culture Collection (Manassas, VA, USA). Trizol reagent was purchased

from Life Technologies (Carlsbad, CA, USA). All other reagents and solvents were analytical or HPLC grade and used without further purification.

Experimental protocol

Capan-2 pancreatic cancer cells, a human adenocarcinomatous adherent cell line, was chosen not only because it represents the aggressive type of pancreatic cancer (20, 44, 45), but also, it expresses several influx and efflux proteins including MRP1, MRP5, and P-gp (24, 46). This cell line has been conventionally used as a model to study alteration in expression of the efflux proteins (44, 46). Cells were cultured in 75 cm^2 flasks (37 °C under 95:5 air: CO_2) and the recommended media consisted of McCoy's 5A medium supplemented with fetal bovine serum (5% v/v). Media was changed every 2 days. The Capan-2 cells with 70% confluency and total viable cells averaged ~ 2.54 x 10^6 with greater than 98% viability, were rinsed briefly with Ca^{+2}/Mg^{+2} free DPBS and 1-2 mL of 0.25% (w/v) trypsin-0.53 mM EDTA at room temperature. Following cell detachment, fresh culture media was added and the cells were seeded into 12-well plates at a density of ~200,000 cells per well. When the cells were 70-80% confluent, the media was removed and 1 mL of 50 nM solutions of calcitriol, or 1µM solutions of BHT, BHA, or silibinin were added and the wells placed into an incubator at 37 °C (n = 4). This step is being referred as "pre-treatment" in this study. The selected concentrations were within the range of *in vivo* concentrations reported for each of these compounds (47-49). Once the cells were pre-treated for 24 h, the extracellular liquid was removed, and cells were rinsed once with 1 mL DPBS. Afterwards, a 1 mL solution of gemcitabine at a concentration of 1.5 µM was added and incubated for 24 h. Subsequently, the cells were rinsed once with 1 mL DPBS and were lysed by adding either 150 µL of Cellytic M for 15 min, or 500 µL of Trizol for 5 min and stored at –70 °C until further analysis. Controls for this study were "treated-control" (treated only with 1.5µM gemcitabine) and "control" (treated neither with gemcitabine nor with calcitriol, BHT, BHA or silibinin). The "control" samples served as an indicator of the basal levels of activity/expression.

Drug uptake determination

The concentration of gemcitabine was quantified using a UPLC equipped with a tandem quadrupole detector for integrated UPLC/MS/MS quantitation, and a UPLC BEH C_{18} column (2.1 × 150 mm, 1.7 µm) as the solid phase (Waters, Milford, MA, USA). Mobile phase consisted of 0.1% acetic acid in water (solvent A) and acetonitrile (solvent B). The flow rate was 100 µL/min and elution was accomplished at ambient temperature by keeping 90% A and 10% B for 1.5 min, followed by a linear gradient to 90% B and 10% A over 1.5 min, after which the system was returned to the initial condition in 30 seconds. The MRM transition of m/z 264.04 -> 112.0 was chosen for gemcitabine assay. Source temperature was set at 150 °C and cone voltage at 30 V. The integration of the output was handled by the Empower software (Waters, Milford, MA, USA).

For the uptake study, cell lysates incubated with Cellytic M, were injected (20 µL) directly into the UPLC. Gemcitabine concentration was determined by calculating the area under the eluted peak to that of a standard curve. The standard curve was constructed with 12 standard solutions (in CellyticM) in the range 0.125 - 800 ng/mL (r^2 > 0.99). The amount of gemcitabine uptake in picograms (pg) per mg protein was plotted.

Real Time Polymerase Chain Reaction (RT-PCR)

Sample preparation. Total RNA from cancerous cells was isolated with Trizol reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. This was followed by spectrophotometric quantitation of the isolated RNA.

Quantifying gene expression. Using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), cDNA was synthesized from 1.5 µg total RNA samples with the random primers scheme according to the manufacturer protocol. The mRNA expression for genes encoding ABCB1 (P-gp), SLC29A1 (ENT1), ABCC1 (MRP1), and ABCC5 (MRP5) were measured by RT-PCR in a CFX96 Touch real-time PCR detection system (Bio-Rad laboratories, Hercules, CA, USA) using fluorescent TaqMan technology (Life Technologies, Carlsbad, CA, USA). The assays ID of the quantified genes were: ABCB1 (Hs00184500_m1), SLC29A1 (Hs01085704_g1), ABCC1 (Hs01561502_m1), ABCC5 (Hs00981087_m1), and 18s rRNA (Hs99999901_s1) as a control. PCR data were analyzed using the delta delta CT method (ΔΔCT).

Western blotting

Sample preparation: The Cellytic M treated cell lysates were thawed and their protein contents determined using Bradford protein assay (Thermo Scientific, Waltham, MA, USA). Appropriate volumes of each sample were mixed with ice-cold tris buffer/protease inhibitor cocktail (19:1) to prepare a protein concentration of 1mg/mL.

Measurements of protein expression: The density of P-gp, ENT1, MRP1, and MRP5 proteins were measured by western blotting. Briefly, samples were boiled at 100 °C and 20 µL of each sample were loaded onto a precast 4–20% TRIS–HCl gel (Bio-Rad laboratories, Hercules, CA, USA) and SDS-gel electrophoretic separation was carried out for 80 min at 125 V. The separated proteins were transferred onto a nitrocellulose membrane and incubated 30 min at room temperature with Starting Block buffer (Thermo Scientific, Waltham, MA, USA). After washing the membranes with the buffer, the available primary antibodies were added at the recommended dilution by the manufacturer. For P-gp detection, the primary antibody was rabbit monoclonal antibody to P-gp, whereas, rabbit polyclonal antibody to ENT1 was used for ENT1 detection. Mouse monoclonal to MRP1 and rat monoclonal to MRP5 were used for MRP1 and MRP5, respectively. Rabbit polyclonal antibody to β-actin was utilized as the primary antibody for detection of endogenous control protein (β-actin). Following addition of the primary antibodies, the secondary antibodies namely horseradish peroxidase-conjugated goat polyclonal antibody to rabbit IgG, rabbit polyclonal antibody to mouse IgG, or rabbit polyclonal antibody to rat IgG were added to the membranes. SuperSignal west pico chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) was used in order to produce a luminescent derivative of the favorable protein to be detected and quantified by chemiluminescence. Blots were imaged with a Chemidoc XRS (Bio-Rad laboratories, Hercules, CA, USA) and net band intensity units were measured with Image Lab image analysis software (Bio-Rad laboratories, Hercules, CA, USA). All the primary and secondary antibodies were obtained from Abcam (Abcam Inc., Cambridge, MA, USA). The photometric density of the blots (representing the amount of the protein) for each protein was corrected for loading variations by taking the ratio to the control housekeeping protein (β-actin).

Measurement of ATPase activity of the cell membrane efflux proteins

Effects of pre-treatments on the efflux proteins basal ATPase activity of the cell membrane transporters were investigated and compared to the control cells. Enrichment and extraction of the integral membrane proteins and membrane-associated proteins from the cultured cells were achieved by using membrane protein extraction kit (Mem-PER Plus, Thermo Scientific, Waltham, MA, USA). The protein contents of the samples were determined using the Bradford method for protein assay (Thermo Scientific, Waltham, MA, USA). ATPase activity was measured using a previously described method (50). Briefly, to investigate the effect of incubation of gemcitabine on the enzyme activity, a suspension of membrane protein (1 mg/mL) was prepared in tris-buffer (pH 7.4, 50 mM) and 30 µL of the prepared membrane suspension was added to the appropriate wells of the 96 well plates. Because protease and phosphatase inhibitors can interact with this assay, none was added to the samples. In order to determine the sodium orthovanadate (Na_3VO_4) insensitive ATPase activity, to a separate set of samples (30 µL), 1 µL of 60 mM sodium orthovanadate solution was added. Plates were pre-incubated for 5 min at 37 °C. The ATPase reaction was started by addition of 10 µL of 25 mM Mg-ATP solution. The plates were incubated at 37 °C for 20 minutes and the reaction was stopped by adding 50 µL of 5% sodium dodecyl sulfate (SDS) solution to each well.

A colorimetric method was used to detect inorganic phosphate (Pi) liberation in the assay medium. The color reagent consisted of ammonium molybdate in a 15 mM mixture of zinc acetate /10% ascorbic acid (1:4) added (200 µL) to each well and incubated at 37 °C for 25 minutes and the absorbance measured at 630 nm. The liberated Pi was determined using the absorbance of phosphate calibration curve and the results were calculated based on the amount of protein and incubation time.

Cell Viability Assay

Viability of the cells was determined using the Promega luminescent cell viability assay (Promega, Madison, WI, USA). Luminescence was measured using BioTek Synergy2 plate reader (BioTek Instruments, Winooski, VT, USA). Cells at a density of ~20,000 cells per well were transferred to 96 well cell culture plates and after being at 70-80% confluence, they were pre-treated with calcitriol, BHT, BHA or silibinin followed by gemcitabine

treatment as described in the "experimental protocol" section. At the end of the experimental time, the viability of the cells in different treatment concentrations was determined and compared to the values of the control groups. All experimental incubation conditions were performed in triplicates. Erythrosin B (American Type Culture Collection, Manassas, VA, USA) staining method was used as an alternative method to confirm the cell viability results. Cell death was estimated as [100 - percentage of the viable cells].

DATA ANALYSIS

Data are presented as mean ± SD. The influence of the pre-treatment conditions on the expression of mRNAs and proteins, gemcitabine uptake, ATPase activity, and cell death were investigated by utilizing one-way ANOVA analysis (followed by Tukey's post-test). Statistical analyses were carried out using Prism software (GraphPad Software Inc., San Diego, CA, USA) at a significance level of $p < 0.05$.

RESULTS

UPLC analysis and gemcitabine uptake determination

Figure 1, demonstrates the amount of gemcitabine uptake after 24 h. A significant difference in the amount of gemcitabine uptake was observed in all groups as compared to the treated-control group. Pre-treatment with calcitriol resulted in a significant increase in gemcitabine uptake, whereas BHA, BHT, and silibinin reduced gemcitabine uptake significantly as compared to the treated-control group. The lowest gemcitabine uptake was observed after BHA pre-treatment. The stability of gemcitabine in the medium at 37° C for 72 h was investigated and confirmed to be over 98% within 72h.

Real Time Polymerase Chain Reaction (RT-PCR)

The changes in mRNA expression of the influx and efflux proteins are presented in Figure 2. Treatment with gemcitabine increased mRNA expression of ENT1. Pre-treatment with calcitriol, had a significant additive effect on mRNA expression of ENT1, whereas pretreatment with either BHT or silibinin, did not appear to change the expression as compared to the treated-control samples. Interestingly, pre-treatment with BHA significantly decreased mRNA expression of ENT1 as compared to the treated-control group (Figure 2a). mRNA expression of MRP1 and MRP5 were significantly increased by gemcitabine treatment. Pre-treatment with BHT, silibinin, and BHA had an additive effect and significantly increased expression as compared to the treated-control group; in general, the effect of BHA was more significant. On the other hand, pre-treatment with calcitriol, significantly inhibited expression of MRP1 and MRP5 mRNA as compared to treated-control samples (Figure 2b-2c). Pgp mRNA expression was induced by gemcitabine. Pre-treatment with Calcitriol did not show a significant effect on Pgp mRNA expression, whereas pre-treatment with BHA, Silibinin, and BHT significantly induced Pgp mRNA expression (Figure 2d).

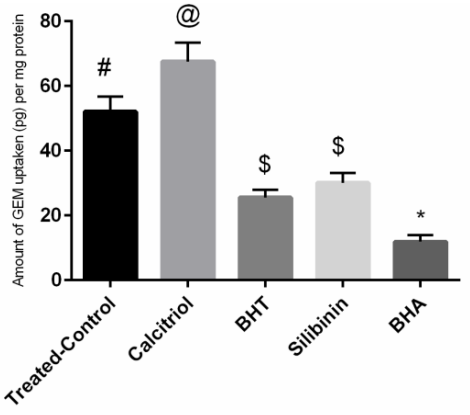

Figure 1. Amount of uptaken gemcitabine in treated-control cells and after pre-treatment with calcitriol, BHT, silibilin, and BHA followed by gemcitabine treatment. Different characters denote significant differences between the groups (p value < 0.05).

Western blotting and protein expression

The relative expression of the proteins is presented in Figure 3. Treatment with gemcitabine significantly induced expression of all studied influx and efflux proteins. Pre-treatment with calcitriol, silibinin, or BHT did not significantly affect expression of ENT1 protein (Figure 3a). However, there was a trend toward higher ENT1 expression in calcitriol pre-treated samples. BHT, on the other hand decreased the relative ENT1 expression significantly (Figure 3a).

During the course of this study, we realized that the calcitriol had the opposite effect on expression of efflux proteins as compared to BHT, BHA, and silibinin. BHT, silibinin, and BHA significantly

increased the protein expression of MRP1 and MRP5 as compared to the treated-control group; BHA showed a more pronounced effect on expression induction. In contrast, calcitriol significantly inhibited expression of MRP1 and MRP5 efflux proteins (Figure 3b-3c). The expression of Pgp was not significantly changed after pre-treatment with any of the studied compounds (Figure 3d).

Measurement of ATPase activity of the cell membrane efflux proteins

ATPase activities of the cell membrane efflux transporters are presented in Figure 4. We observed that an increase or decrease in basal ATPase activity of the efflux proteins reasonably correlated with induction of the protein expressions. It was also indicated that the expressed proteins are functionally active. While gemcitabine exposure induced the activity level; pre-treatment with BHT, silibinin, and BHA further increased basal activity. On the other hand, calcitriol significantly inhibited induction and kept ATPase activity at basal levels.

Cell Viability

The percent cell viability is presented in Figure 5. Treatment with gemcitabine resulted in an average of 29% cell death after 24 h. Pre-treatment with BHT and silibinin had no significant effect on the cell death rate. Average cell death after BHA pre-treatment was approximately 84%, which was significantly more as compared to the treated-control cells. Interestingly, pre-treatment with calcitriol resulted in a significant increase in cell deathwhere on average only 51% of the cells survived.

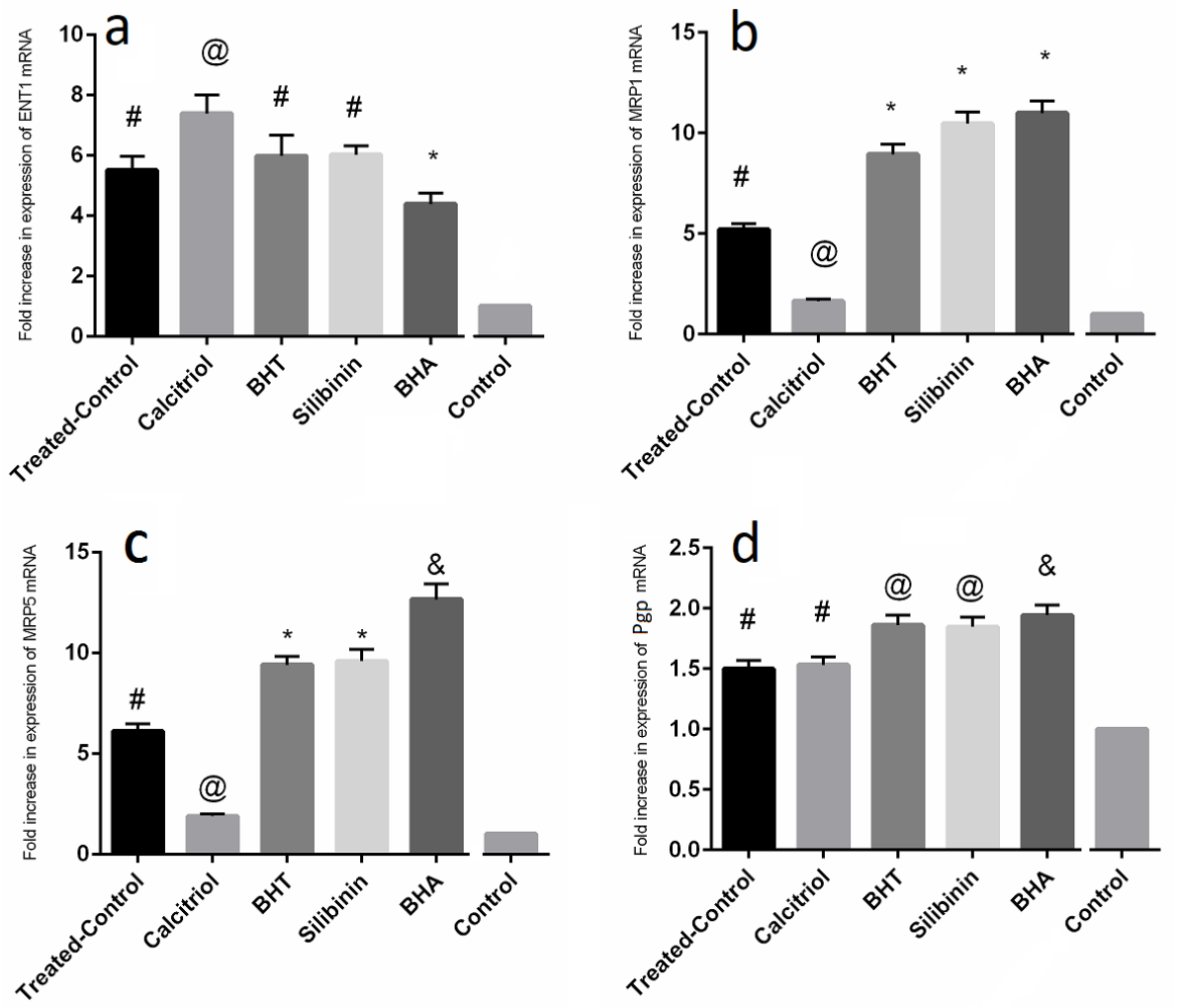

Figure 2. Fold increase in expression of mRNA expression after pre-treatment with calcitriol, BHT, silibilin, and BHA followed by gemcitabine treatment and in the control samples (2a-2d). Different characters denote significant differences between the groups (p value < 0.05).

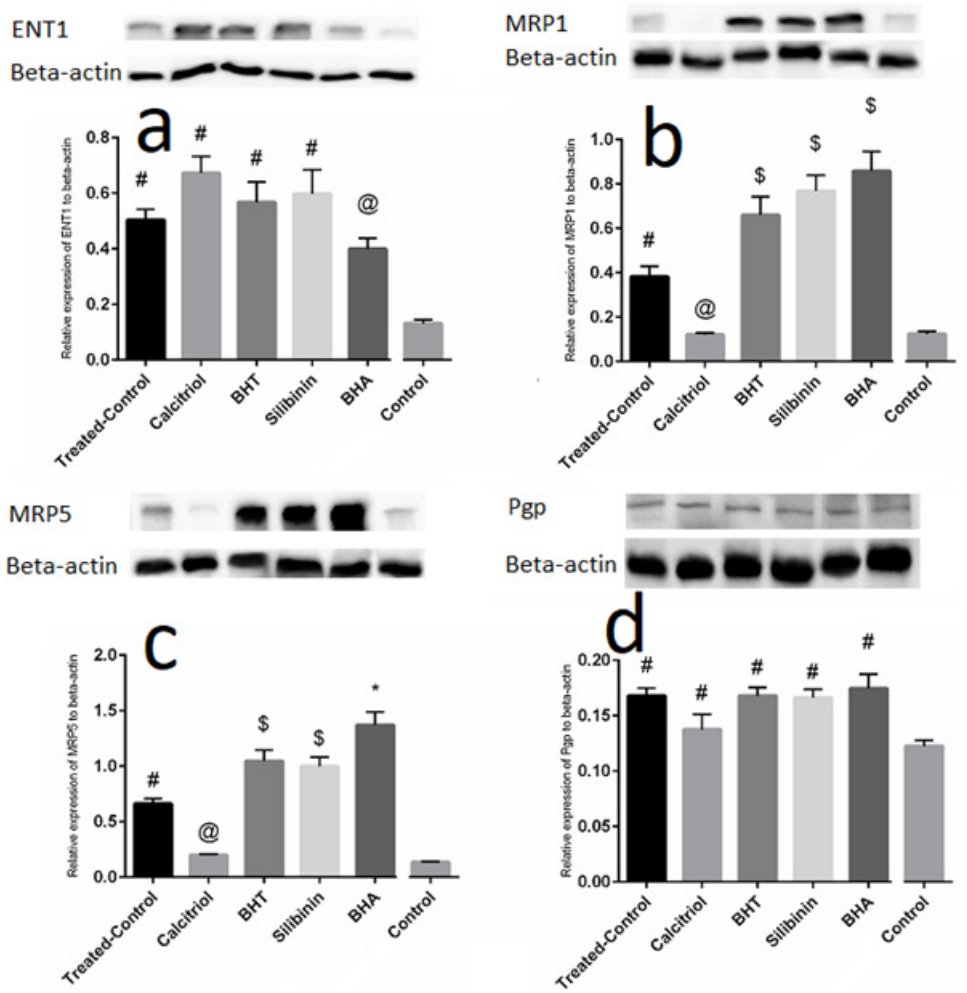

Figure 3. The relative expression of the proteins after pre-treatment with calcitriol, BHT, silibilin, and BHA followed by gemcitabine treatment and in the control samples (3a-3d). Different characters denote significant differences between the groups (p value < 0.05).

DISCUSSION

Capan-2 pancreatic cancer cells, similar to many other type of cancerous cells, utilize various cellular mechanisms such as microparticle formation (51), and induction of efflux proteins against anticancer drugs (24). Several studies have shown that the resistance of pancreatic cancer cells to chemotherapeutics including gemcitabine, is the result of reduced cellular uptake via influx proteins (12) or their efficient removal from cells by efflux proteins (15, 24). Synthetic antioxidants such as BHA and BHT are widely used in pharmaceutical products as well as in the food industry to protect susceptible chemicals against oxidation (27, 28). Calcitriol is the active metabolite of vitamin D playing an important role in regulation of intestinal calcium absorption, and metabolism of bone and minerals (38, 39). Silibinin is the major flavonoid of silymarin, a standardized extract of milk thistle seeds that conventionally has been used for treatment of liver diseases. Although there are few studies to investigate the potential impact of these chemicals in cancer therapy, currently our knowledge of the impact of these compounds on the expression of efflux and influx proteins in pancreatic cancer cells is very limited. Hence, investigation of the effect of

pre-treatment with these commonly used vitamins and anti-oxidants on expression profiles of efflux and influx proteins are considered very important, as there are no specific guidelines regarding the use of these compounds prior to or during the chemotherapy. As any alteration of the efflux and influx protein expression, may result in variations of anti-cancer drug uptake and consequently cell death rate, we investigated the expression of influx and efflux proteins in pre-treated capan-2 cells with calicitriol, silibilin, BHT, and BHA. Pre-treatment followed by gemcitabine treatment and subsequent drug uptake and cellular death rate were evaluated.

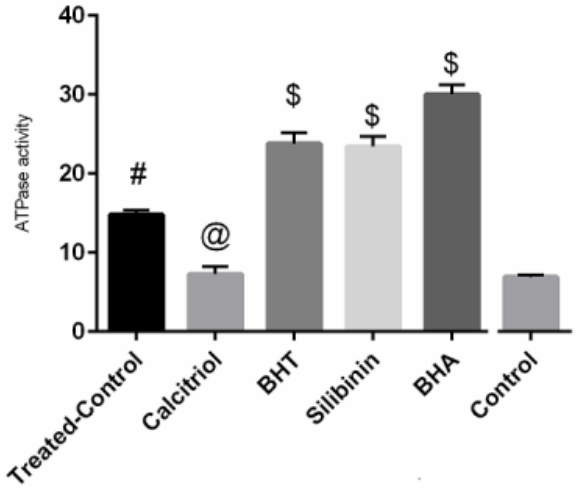

Figure 4. Membrane ATPase activity after pre-treatment with calcitriol, BHT, silibilin, and BHA followed by gemcitabine treatment and in the control samples. Different characters denote significant differences between the groups (p value < 0.05).

The data from this investigation demonstrated that gemcitabine uptake increased significantly by pre-treatment with calcitriol, whereas BHA, BHT, and silibinin reduce gemcitabine uptake dramatically (Figure 1). These changes in the uptake rate were at least partially attributed to the changes in expression of the influx and efflux proteins, as the protein expression correlated well with the uptake; BHT, silibinin, and BHA increased protein expression of MRP1 and MRP5 and hence decreased the overall uptake, whereas calcitriol significantly inhibited expression of MRP1 and MRP5 efflux proteins, which resulted in increased uptake.

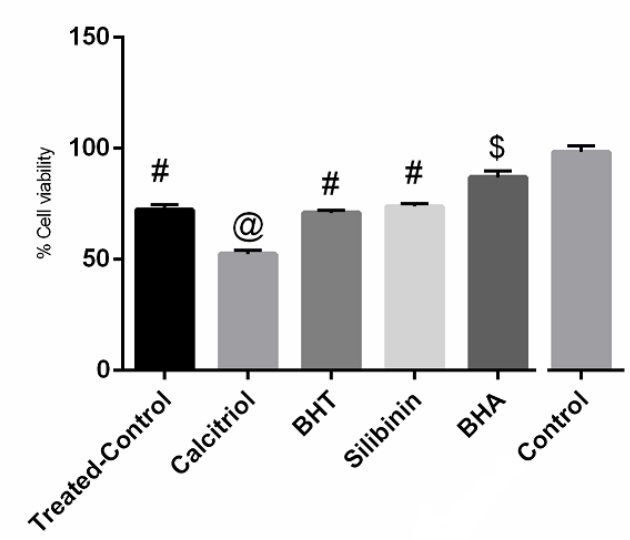

Figure 5. Cell viability (%) after pretreatment with calcitriol, BHT, silibilin, and BHA followed by 24 h gemcitabine treatment and in the control samples. Different characters denote significant differences between the groups (p value < 0.05).

BHA showed a more pronounced effect in the protein expression induction as compared to BHT and silibinin. Expression of Pgp was not significantly changed after pre-treatment with any of the studied compounds (Figure 3). In general, the decline or increase of the uptake rate of gemcitabine is the result of the combined influence of all factors involved in the disposition of gemcitabine, which includes formation of phosphorylated metabolites, deactivation through deamination in conjunction with overexpressed efflux proteins, and their effective removal of the parent compound and possibly metabolites from the cells. Hence, the data presented in this study is the sum of the combined influence of all factors.

The correlation between the overall ATPase activity of membrane extract and the efflux protein functionality has been established in several studies (24, 52-54). The observed induction of the membrane ATPase activity indicated that the induced efflux proteins are functionally active (Figure 4).

In our previous study, the time line of expression of the studied efflux and influx proteins and their mRNA expression had been shown (24). In this study, expression of mRNAs correlated reasonably well with the levels of the corresponding proteins of the influx and efflux pumps (Figure 2).

Capan-2 cells appeared to be relatively resistant to gemcitabine therapy and only 29% cell death occurred after 24 h exposure to gemcitabine. Pre-treatment with BHT and silibinin had no significant effect on the cell death rate. BHA pre-treatment significantly reduced the cell death rate. Interestingly, pre-treatment with calcitriol resulted in a significant increase in cell death where on average only 51% of the cells survived (Figure 5).

In conclusion, pre-treatment of the pancreatic cancer cells with calcitriol significantly increases gemcitabine cellular uptake and consequently increases the rate of cell death, whereas BHA significantly reduces gemcitabine uptake and decreases the cell death rate, which may, at least in part, be attributed to an alteration of expression of efflux and influx proteins. Further clinical studies are warranted to relate the findings of this study to real life clinical practice settings.

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