Cancer Chemother Pharmacol (2003) 52: 235–246
DOI 10.1007/s00280-003-0628-6

ORIGINAL ARTICLE

Shadan Ali · Olivia Aranha · Yiwei Li
George R. Pettit · Fazlul H. Sarkar · Philip Agop Philip

Sensitization of human breast cancer cells to gemcitabine by the protein kinase C modulator bryostatin 1

Received: 6 November 2002 / Accepted: 19 March 2003 / Published online: 14 June 2003

Abstract *Purpose*: Protein kinase C (PKC) plays an important role in cell proliferation, differentiation, and apoptosis. The interaction between the PKC modulator bryostatin 1 (BRYO), and gemcitabine in human breast cancer MCF-7 and MDA-MB-231 cells and in the non-transformed MCF-10A human breast epithelial cells was investigated. *Methods*: Immunoblotting was used to determine the expression of PKC isoenzymes and proteins involved in the cell cycle and apoptosis. MTT, ELISA and flow cytometry assays were used to determine cell survival. *Results*: Treatment of cells with BRYO 200 n*M* resulted in a significant downregulation of cytoplasmic PKC in all three cell lines. However, the expression of membranous PKC was differentially affected in these cells. BRYO (1–200 n*M*) had no significant effects on cell viability in any of the cell lines. Nevertheless, BRYO significantly enhanced the antiproliferative and apoptotic effects of gemcitabine in the MCF-7 and MDA-MB-231 cells, but not in the MCF-10A cells. This was associated with significant reduction in the bcl-2/bax ratio. There was a significant upregulation of p53, $p21^{waf1}$, and p27 in MCF7 and MCF-10A cells treated with the combination of gemcitabine and BRYO compared to gemcitabine-treated cells. *Conclusions*: The potentiation of the effect of gemcitabine by BRYO was demonstrated in MCF-7 and MDA-MB-231 cells and was associated with a specific pattern of PKC modulation. Further investigation of the role of specific isoforms of PKC in the downstream molecular events of gemcitabine-induced cytotoxicity is warranted.

Keywords Bryostatin 1 · Gemcitabine · Breast neoplasms · Protein kinase C · Cell cycle

S. Ali · O. Aranha · Y. Li · F. H. Sarkar · P. A. Philip
Karmanos Cancer Institute, Wayne State University,
Detroit, MI 48201, USA

G. R. Pettit
The Institute of Cancer Research, Arizona State University,
Tempe, AZ, USA

P. A. Philip (✉)
Division of Hematology and Oncology,
Barbara Ann Karmanos Cancer Institute,
Wayne State University,
501 Hudson Building, 3990 John R Street,
Detroit, MI 48201, USA
E-mail: Philipp@karmanos.org
Tel.: +1-313-7458039
Fax: +1-313-9662944

Introduction

Approximately 44,000 women die of breast cancer per year in the USA despite the introduction of newer anticancer drugs (Jemal et al. 2002). Failure of therapy is attributed to the resistance of breast cancer cells to cytotoxic drugs. Mechanisms of drug resistance have been extensively investigated in recent years. The dysregulated signaling in cancer cells makes it a potential target to improve the apoptotic activity of the existing cytotoxic drugs (Jarvis and Grant 1999).

Protein kinase C (PKC) is a family of homologous serine/threonine protein kinases that transduce signals linked to diverse cellular processes that include proliferation, differentiation, and apoptosis (Deacon et al. 1997; Stabel 1994). The PKC family comprises 12 isoforms subdivided into three major classes based on their cofactor requirements for activation. There is also evidence that PKC activity is differentially regulated in normal tissues and their malignant counterparts, indicating a role for aberrant regulation of PKC in carcinogenesis. In general, PKC-mediated signaling pathways are more activated in malignant compared with normal breast tissue (Gordge et al. 1996; O'Brian et al. 1989). Although molecular cloning has demonstrated the existence of multiple PKC isoforms, the majority of these have not yet been well characterized as to their functions in carcinogenesis and cellular response to anticancer drugs. The heterogeneous and tissue-selective expression of PKC isoforms may also explain the marked variability of cellular responses to PKC modulation.

Gemcitabine (2′,2′-difluorodeoxycytidine) is a deoxycytidine analog that has significant antitumor activity against several human malignancies including breast cancer (Carmichael et al. 1995). Gemcitabine is more potent than cytosine arabinoside with a much broader anticancer activity against non-hematological cancers. Incorporation of the triphosphate metabolite of gemcitabine into newly formed DNA strands inhibits DNA synthesis (Huang and Plunkett 1995).

There is growing evidence that modulators of PKC and other signaling molecules may have limited therapeutic utility as anticancer agents when used alone (Zonder et al. 2001). However, these agents may be of value in the treatment of cancer by enhancing the antiproliferative and proapoptotic effects of conventional cytotoxic drugs. Several drugs have been developed that modulate PKC activity and may, therefore, be of therapeutic potential in human cancers (Philip and Harris 1995). Of those, the macrocyclic lactone, bryostatin 1 (BRYO), has been the first to complete phase I clinical studies (Philip et al. 1993; Prendiville et al. 1993) and currently is in phase II clinical trials in several tumor types. Unlike conventional cytotoxic therapy, the dose-limiting toxicities to BRYO are non-hematological allowing its use in combination with conventional cytotoxic drugs with minimal added hematologic toxicity.

The key to further development and use of selective PKC modulators depends on our understanding of the involvement of specific PKC isoforms in cytotoxic drug resistance/sensitivity. The rationale for the present study was the synergy between BRYO and cytosine arabinoside, an analog of gemcitabine, against human leukemia cells (Beltran et al. 1997; Grant 1997; Grant and Jarvis 1996). In addition, gemcitabine cytotoxicity may be influenced by PKC activity (Cartee and Kucera 1998). We therefore hypothesized that the modulation of PKC activity in human breast cancer cells by BRYO would increase the sensitivity of these cells to gemcitabine by enhancement of apoptosis. To test this hypothesis, we studied the effect of the combined treatment of BRYO and gemcitabine on the proliferation and survival of MCF-7, MDA-MB-231, and MCF-10A human breast cells.

Materials and methods

Chemicals and reagents

BRYO and gemcitabine were generous gifts from the Arizona State University Cancer Research Institute (Tempe, Ariz.) and Lilly Research Laboratories (Indianapolis, Ind.), respectively. 3-[4,5-Dimetylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), isopropanol, and dimethylsulfoxide (DMSO) were acquired from Sigma Chemicals (St. Louis, Mo.). Monoclonal antibodies against human PKC-α, -βI, -βII, and -ϵ were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Propidium iodide was obtained from Sigma Chemicals. Cell culture media were purchased from Gibco-BRL (Grand Island, N.Y.).

MCF-7, MDA-MB-231, and MCF-10A cells in culture

Human breast cancer cells MCF-7, MDA-MB-231 and breast epithelial untransformed MCF-10A cells were used in this study. MDA-MB-231 and MCF-7 cells were grown as a monolayer cell culture in DMEM/F12 (1:1) with 10% fetal bovine serum. The latter was also supplemented with HEPES 10 n*M*, estradiol 0.5 n*M*, human insulin 10 μg/ml, and penicillin/streptomycin 1% at 37°C in a humidified atmosphere of 5% CO_2/95% air. MCF-10A cells were grown in DMEM/F12 medium supplemented with insulin 10 μg/ml, epidermal growth factor 20 ng/ml, cholera toxin 100 ng/ml, hydrocortisone 0.5 μg/ml, and 5% equine serum at 37°C.

MTT assay

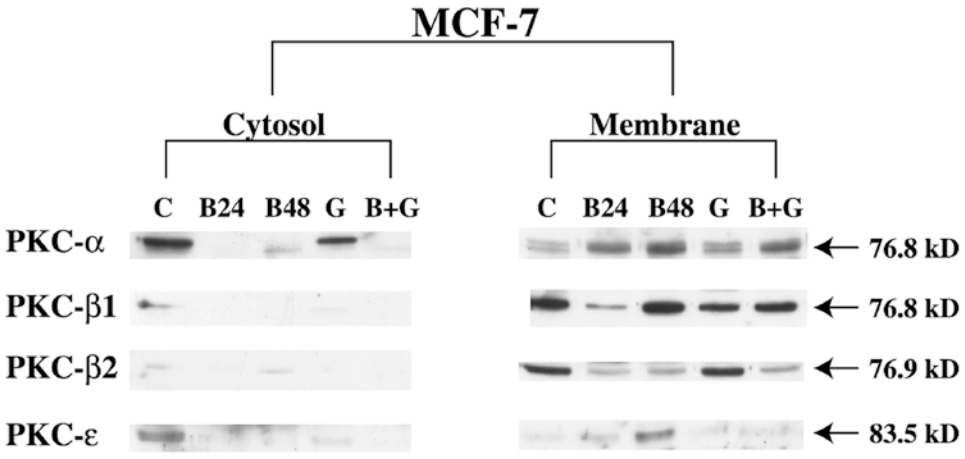
Viable cell growth was determined by the standard MTT reduction assay with slight modifications (Carmichael et al. 1985). Cells were plated (10–20,000/well) in a 96-well plate and incubated overnight at 37°C. BRYO was dissolved in DMSO and added to the cell culture medium at a volume/volume concentration not exceeding 0.1%. The effects of BRYO and gemcitabine on MCF-7, MDA-MB-231 and MCF-10A cells were studied using different sequences of drug administration, including concomitant incubation with the two drugs. The assay was performed in triplicate for each concentration used. After the required drug treatment time, aliquots of 100 μl MTT (1 mg/ml) were added to each well followed by incubation for 2 h at 37°C. The supernatant was removed and isopropanol 200 μl was then added. The color intensity was measured using a kinetic microplate reader (Molecular Devices, Sunnyvale, Calif.) at 595 nm. DMSO-treated cells were assigned a value of 100%. The linearity of color intensity relative to cell number within the range expected in the study was determined at the outset.

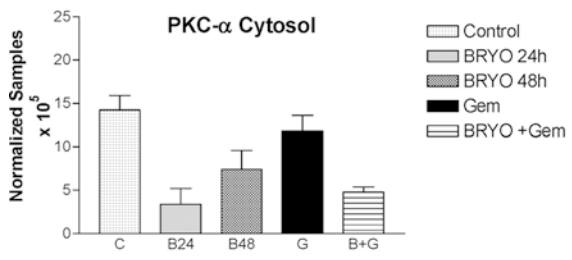
Apoptosis assay using ELISA

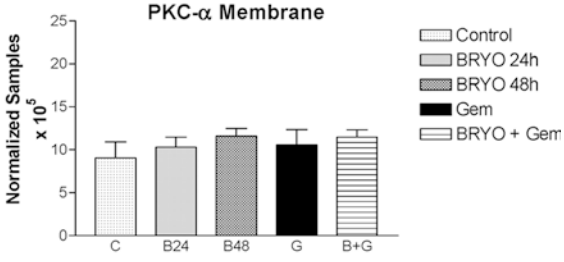
The Cell Death Detection ELISA^{Plus} kit (Boehringer-Mannheim, Indianapolis, Ind.) was used to detect apoptosis. The assay is based on a photometric enzyme-immunoassay procedure for the qualitative and quantitative determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). The assay utilizes anti-histone biotin antibodies that bind to H2A, H2B, H3 and H4 histones and anti-DNA POD antibodies that react with single- and double-stranded DNA. Exponentially growing cells were treated with BRYO (200 n*M*), gemcitabine (10 n*M*), or the combination of both drugs for 24 h, 48 h, and 72 h. The supernatant collected from the wells was centrifuged and the floating cells were included in the assay. Approximately 200 μl lysis buffer was added to the attached cells followed by incubation at room temperature for 30 min. The plates were centrifuged at 200 *g* for 10 min and 20 μl supernatant was transferred to a streptavidin-coated microtiter plate. A mixture containing 80 μl anti-histone biotin and anti-DNA peroxidase with incubation buffer was added to the same streptavidin-coated plate followed by incubation for 2 h. After removal of the unbound antibodies by a washing step, the amount of the nucleosomes was quantified by the peroxidase retained in the immunocomplex. The color reaction was measured photometrically with 2,2′-azino-di[3-ethylbenzthiazolin-sulfonate] as the substrate at 490 nm. A positive control supplied by the manufacturer was included in the procedure.

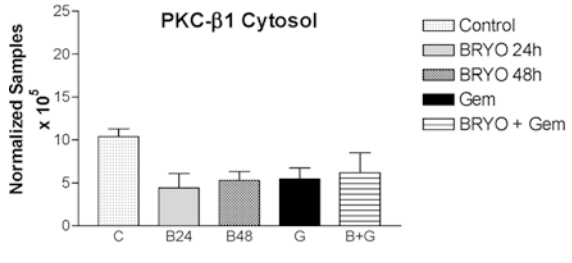
►

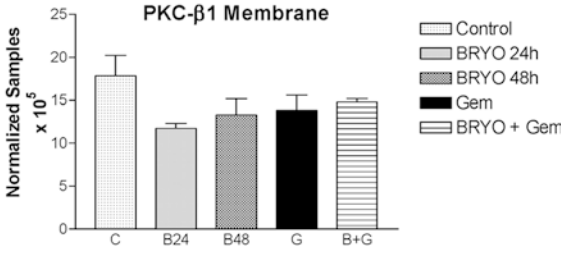
Fig. 1 Representative immunoblots of the effects of BRYO 200 n*M* and gemcitabine 100 n*M* on the expression of PKC-α, -βI, -βII, and -ϵ in human breast cancer MCF-7 cells. Immunoblotting with polyclonal antibodies against respective PKC isoforms was performed to determine protein expression in the membrane and cytosol fractions of the cells. The bidimensional optical density of proteins on the films was quantified and analyzed (*B* bryostatin 1, *G* gemcitabine)

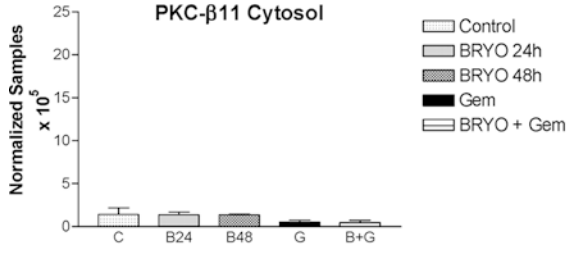

MCF-7
Cytosol
Membrane
C B24 B48 G B+G
C B24 B48 G B+G
PKC-α
PKC-β1
PKC-β2
PKC-ε
76.8 kD
76.8 kD
76.9 kD
83.5 kD

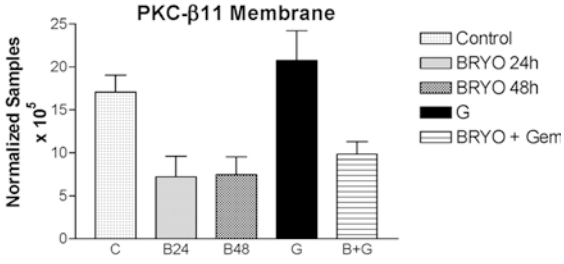

PKC-α Cytosol
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO +Gem
C B24 B48 G B+G

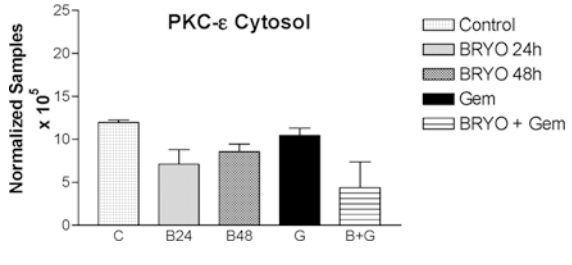

PKC-α Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G

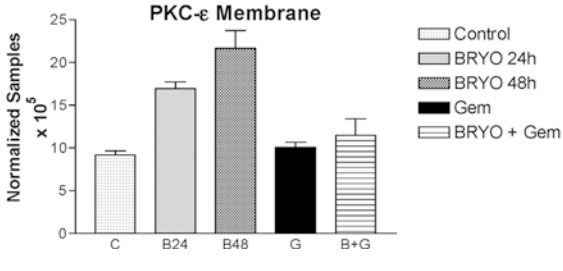

PKC-β1 Cytosol
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G


PKC-β1 Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G


PKC-β11 Cytosol
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G


PKC-β11 Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
G
BRYO + Gem
C B24 B48 G B+G


PKC-ε Cytosol
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G


PKC-ε Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G

Apoptotic cell death by flow cytometry

MDA-MB-231, MCF-7 and MCF-10A cells were treated with BRYO, gemcitabine and the combination for 72 h. Cells were trypsinized and the pellet was suspended in phosphate-buffered saline (PBS) solution. Both positive (heat-killed) and negative (unstained) cells were included in the assay. After adding 100 µl 7-aminoactinomycin (7AAD; Calbiochem, San Diego, Calif.) solution to all cells suspended in PBS, except for the negative control cells, the cells were incubated in the dark for 20 min at 4°C. The cells were pelleted by centrifugation and resuspended in 500 µl PBS/BSA (1:1 ratio). Samples were then analyzed using a FacStar Plus flow cytometer (Becton Dickinson, San Francisco, Calif.) and data processed by Lysis II software (Becton Dickinson).

Immunoblotting for PKC isoenzymes

Expressions of PKC isoforms were determined in the MCF-7, MDA-MB-231 and MCF-10A cells. Cells were plated in T-75 flasks until 80% confluency and were then treated with BRYO, gemcitabine, or the combination. Immunoblot analysis of the PKC isoenzymes (α, βI, βII, ϵ) was performed using antibodies against the different isoenzymes as previously described (Chen et al. 1994). In brief, cells were washed with 10 ml PBS three times and suspended in 500 µl lysis buffer containing Tris-HCl 20 m*M* (pH 7.5), EDTA 2 m*M*, EGTA 0.2 m*M*, leupeptin 10 µg/ml, aprotinin 10 µg/ml, and PMSF 0.2 m*M*. The cells were passed through a syringe several times. Cytosolic and membrane fractions were separated by low- and high-speed centrifugations. An aliquot of each sample (20 µg proteins) was electrophoresed by 10% SDS-PAGE. The proteins resolved on the gel were then electrotransferred onto supported nitrocellulose membranes. The membrane was blocked in 5% dried milk in PBS with Tween 0.05% (PBST) and then incubated overnight with PKC antibodies (1:100 dilution; Santa Cruz Biotechnology) at 4°C. The immunocomplex was then detected with an Amersham ECL or ECL Plus kit (Amersham, Arlington Heights, Ill.). The intensity of the bands was quantified and analyzed using molecular analysis software (Bio-Rad, Hercules, Calif.). A pooled sample was run on every gel as a standard to control for variation between blots.

Immunoblotting for $p21^{WAF1}$, p27, p53, and apoptosis proteins

The influence of drug therapy on the expression of $p21^{WAF1}$, p27, p53, bcl-2, and bax proteins in MCF7, MDA-MB-231, and MCF-10A cells was also determined by immunoblotting. Untreated cells and those treated with different concentrations of gemcitabine and/or BRYO were harvested and collected by centrifugation. Cells were resuspended in Tris-HCl buffer, sonicated for 2×10 s, boiled with an equal volume of 8% SDS for 10 min. and subsequently chilled on ice. Protein concentrations were measured by protein assay reagents (Pierce, Rockford, Ill.). The solution was then subjected to 14% or 10% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Each membrane was incubated with monoclonal antibodies against $p21^{WAF1}$ (1:2000, Upstate Biotechnology, Lake Placid, N.Y.), bcl-2, p53 (1:500, Oncogen, Mass.), bax (1:5000, Biomol, Plymouth Meeting, Pa.), and polyclonal β-actin (1:2000, Sigma, St. Louis, Mo.). Blots were washed with PBST and incubated with secondary antibody conjugated with peroxidase. The signal intensity was then measured using a chemiluminescent detection system (Amersham, Arlington Heights, Ill.). Autoradiograms of the Western blots were scanned with a Gel Doc 1000 image scanner (Bio-Rad) that was linked to a Macintosh computer. The bidimensional optical density of $p21^{WAF1}$, bcl-2, bax, p53 and actin proteins on the films was quantified and analyzed with Molecular Analysis software (Bio-Rad). The ratios $p21^{WAF1}$/actin, bax/actin, bcl-2/actin, and p53/actin were then calculated. The ratio bcl2/bax was also calculated after standardizing against actin expression.

Flow cytometry for cell cycle distribution

MCF-7, MDA-MB-231, and MCF-10A cells were treated with BRYO and/or gemcitabine for 48 h. After the required time, the medium was aspirated and the cells were washed with Hank's buffered salt solution (HBSS). The cells were trypsinized with constant rotation until they were detached from the dishes. The cells were then centrifuged and the resultant pellet was resuspended in 2 ml HBSS with 10% fetal bovine serum. Subsequently, 6 ml 70% ethanol was added dropwise while mixing the samples. Cells were then resuspended in HBSS, and counted. Approximately 2×10^6 cells were recentrifuged and 0.1 ml ribonuclease A (1 mg/ml, Sigma) was added to the pellet. Cells were then incubated at 37°C for 30 min, and 1 ml staining solution (propidium iodide 0.05 mg/ml, Sigma) was then added. Cells were kept cold and protected from light for 30–60 min prior to flow cytometry. Cells were analyzed on a FACS 440 flow cytometer for determination of all phases. Ethanol-fixed peripheral blood lymphocytes were used as external controls. A computer polynomial fit and a manual technique were used to determine the S-phase fractions.

Statistical methods

In each of two experiments, differences in the mean level of apoptosis between drug treatment groups were compared via the two-sample *t*-test. We restricted the hypothesis testing to only comparisons of greatest potential interest. For each of the two experiments, we used the multiple comparisons adjustment procedure of Holm (1979) to control for type I inferential error rate at 0.05 across the set of three *t*-tests performed.

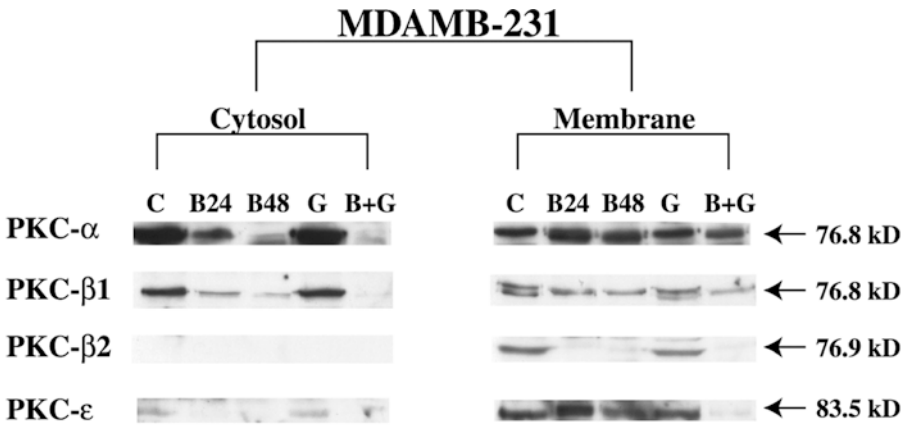
Results

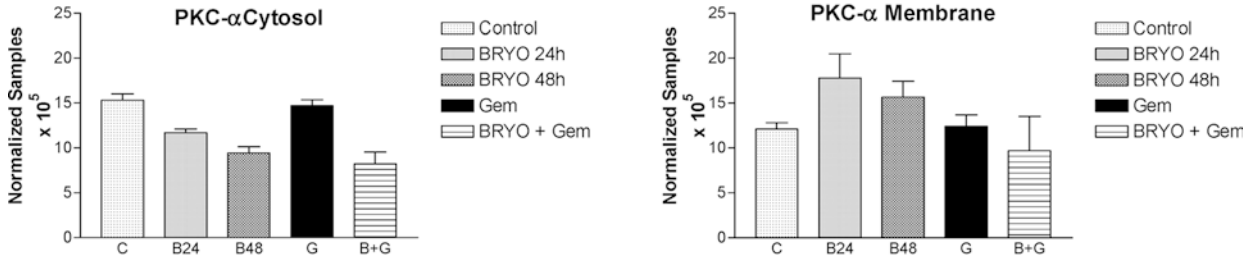
Expression of PKC isoforms in MCF-7, MDA-MB-231, and MCF-10A human breast cells treated with BRYO and/or gemcitabine

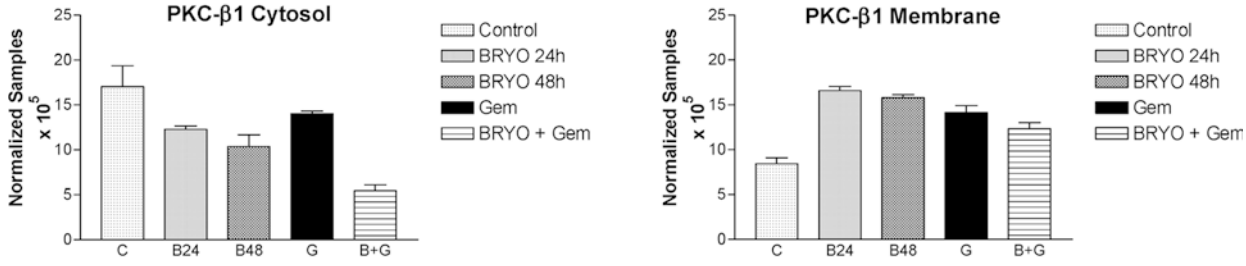
The expression of cytosolic and membranous PKC-α, -βI, -βII, and -ϵ proteins was determined by immunoblotting (Figs. 1, 2 and 3). The expression of these proteins in the untreated cells varied between the three cell lines tested. The expression of the cytosolic PKC-βII in the cytosolic layer was very small in MCF-7 cells and not detectable in the MDA-MB-231 and the MCF-10A cells. The effects of BRYO (200 n*M*) and/or gemcitabine (100 n*M*) for 24 or 48 h on the expression of PKC isoforms in the cytosol and membrane fractions of treated MCF-7, MDA-MB-231, and MCF-10A cells were also determined. It is generally accepted that the translocation of PKC from the cytosol to the membrane results in its activation which is followed by its degradation (Asaoka et al. 1992). BRYO had significantly greater influence on the expression of PKC isoforms than gemcitabine alone. The cytosolic levels of these isoforms,

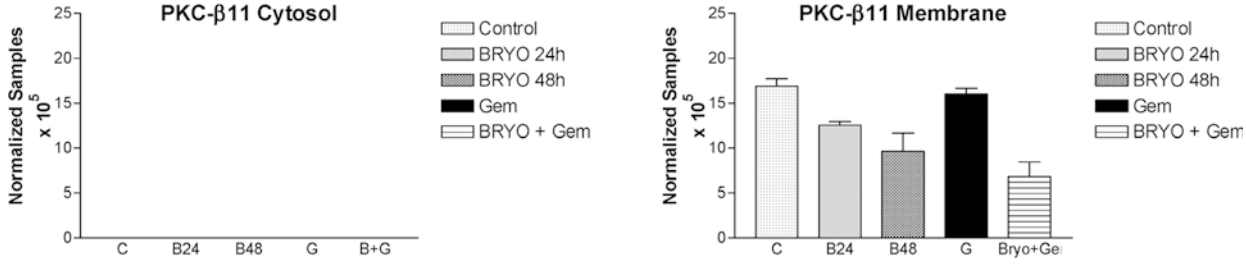
►

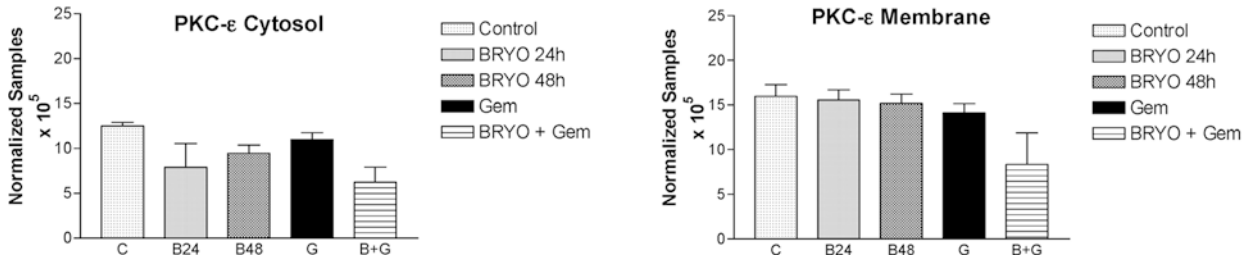
Fig. 2 Representative immunoblots of the effects of BRYO 200 n*M* and gemcitabine 100 n*M* on the expression of PKC-α, -βI, -βII, and -ϵ in human breast cancer MDA-MB-231 cells. Immunoblotting with polyclonal antibodies against respective PKC isoforms was performed to determine protein expression in the membrane and cytosol fractions of the cells. The bidimensional optical density of proteins on the films was quantified and analyzed (*B* bryostatin 1, *G* gemcitabine)

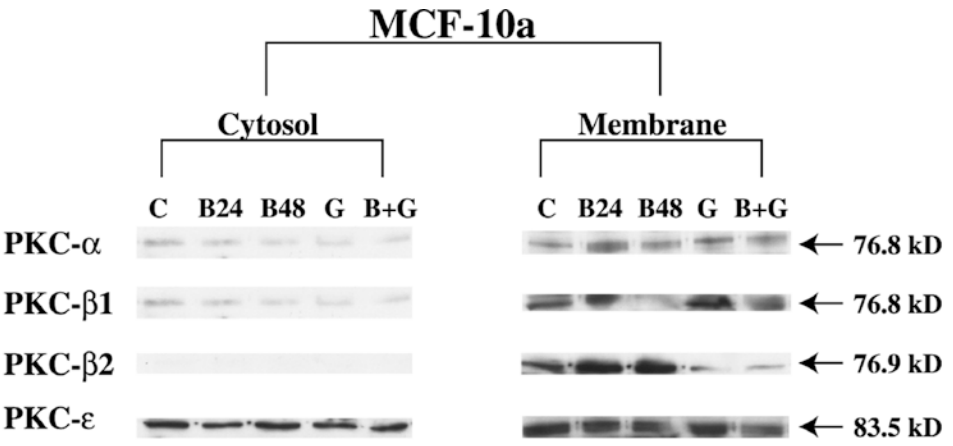

MDAMB-231
Cytosol
Membrane
C B24 B48 G B+G
C B24 B48 G B+G
PKC-α
PKC-β1
PKC-β2
PKC-ε
76.8 kD
76.8 kD
76.9 kD
83.5 kD

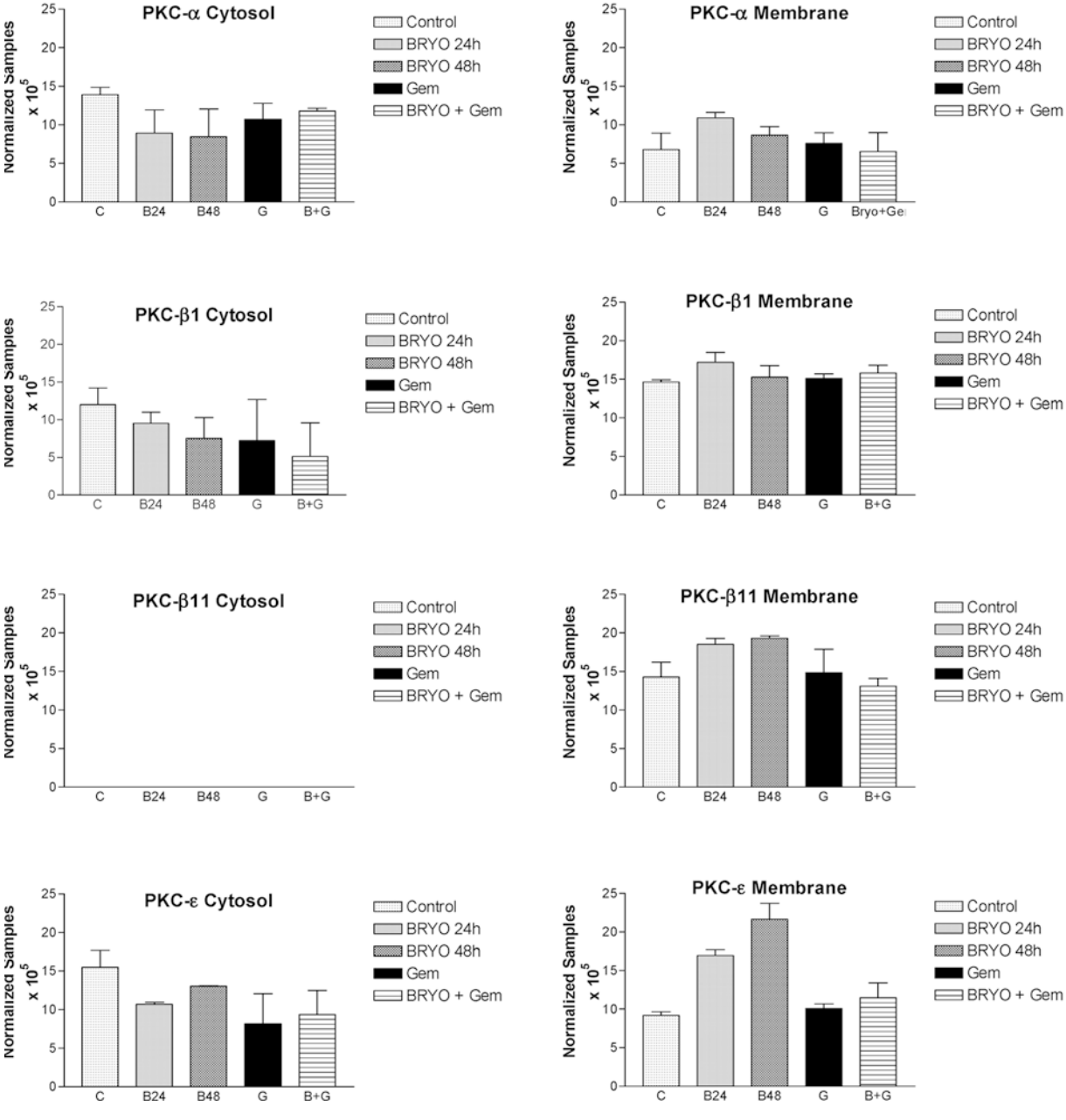

PKC-αCytosol
PKC-α Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G


PKC-β1 Cytosol
PKC-β1 Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G


PKC-β11 Cytosol
PKC-β11 Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G
C B24 B48 G Bryo+Gem


PKC-ε Cytosol
PKC-ε Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G


MCF-10a
Cytosol
Membrane
C B24 B48 G B+G
C B24 B48 G B+G
PKC-α
PKC-β1
PKC-β2
PKC-ε
76.8 kD
76.8 kD
76.9 kD
83.5 kD


PKC-α Cytosol
PKC-α Membrane
PKC-β1 Cytosol
PKC-β1 Membrane
PKC-β11 Cytosol
PKC-β11 Membrane
PKC-ε Cytosol
PKC-ε Membrane
Normalized Samples x 10^5
Control
BRYO 24h
BRYO 48h
Gem
BRYO + Gem
C B24 B48 G B+G
C B24 B48 G Bryo+Ge

◄

Fig. 3 Representative immunoblots of the effects of BRYO 200 n*M* and gemcitabine 100 n*M* on the expression of PKC-α, -βI, -βII, and -ϵ in human breast epithelial MCF-10A cells. Immunoblotting with polyclonal antibodies against respective PKC isoforms was performed to determine protein expression in the membrane and cytosol fractions of the cells. The bidimensional optical density of proteins on the films was quantified and analyzed (*B* bryostatin 1, *G* gemcitabine)

except for PKC-βII, were uniformly reduced by BRYO treatment in the three cell lines. However, the effects of BRYO on the expression of PKC in the membrane varied with respect to the PKC isoform and the cell line tested. PKC-α expression in the membrane fraction was either unchanged (MCF-7) or increased (MDA-MB-231 and MCF-10A). The changes in PKC-βII were very significant for its induction in the MCF-10A cells and downregulation in both cancer cell lines. The induction of the calcium-independent PKC-ϵ in MCF-7 was remarkable: there was a more than twofold increase in this isoform.

Viability of MCF-7, MDA-MB-231, and MCF-10A cells treated with BRYO and/or gemcitabine

Viability of MCF-7, MDA-MB-231, and MCF-10A cells treated with BRYO or gemcitabine alone and in combination was determined by the MTT assay. When compared to DMSO treatment, BRYO did not significantly influence cell viability at concentrations up to 200 n*M* (Fig. 4). However, gemcitabine resulted in a concentration-dependent reduction in MCF-7, MDA-MB-231 and MCF-10A cell viability with a plateau at concentrations greater than 50 n*M* (Fig. 5). Treatment of cells with the combination of BRYO 200 n*M* and increasing concentrations of gemcitabine for 72 h resulted in a significant reduction in the viability of the MCF-7 and MDA-MB-231 cells compared to treatment

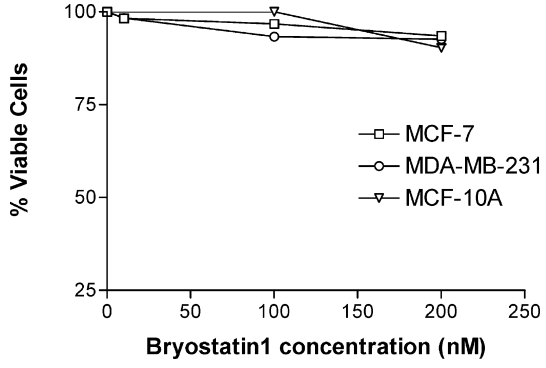


Fig. 4 Human breast cancer MCF-7 and MDA-MB-231 cells and human breast epithelial MCF-10A cells treated with increasing concentrations of BRYO. Cell viability was determined by the MTT assay after a 72-h incubation with BRYO. The results are plotted relative to DMSO-treated control cells and represent the means from at least three separate experiments

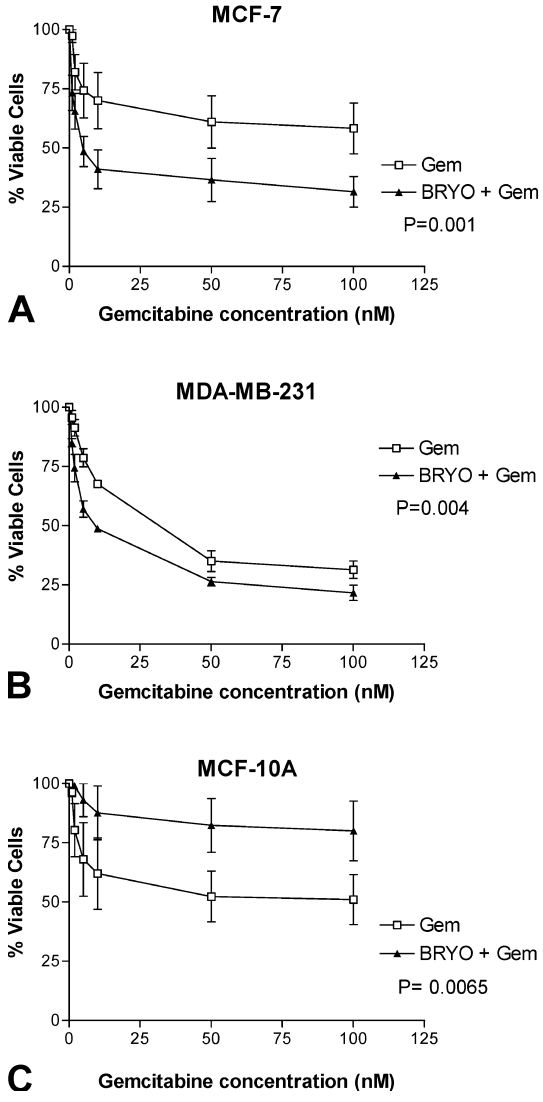


Fig. 5A–C MCF-7, MDA-MB-231, and MCF-10A cells treated with increasing concentrations of gemcitabine. Cell viability was determined by the MTT assay after a 72-h incubation with gemcitabine or BRYO plus gemcitabine. The results were plotted relative to DMSO-treated control cells and represent the means ± SD from at least three separate experiments

with gemcitabine alone (Fig. 5A, B). MCF-7 and MDA-MB-231 cells treated using varying sequences and simultaneous application of both drugs showed no significant difference in survival (data not shown). In contrast, the MCF-10A cells treated with the combination of gemcitabine and BRYO showed no reduction in cell viability compared to cells treated with gemcitabine alone (Fig. 5C).

Induction of apoptosis in MCF-7, MDA-MB-231, and MCF-10A cells

The effect of BRYO and gemcitabine, alone and in combination, on the induction of apoptosis MCF-7,

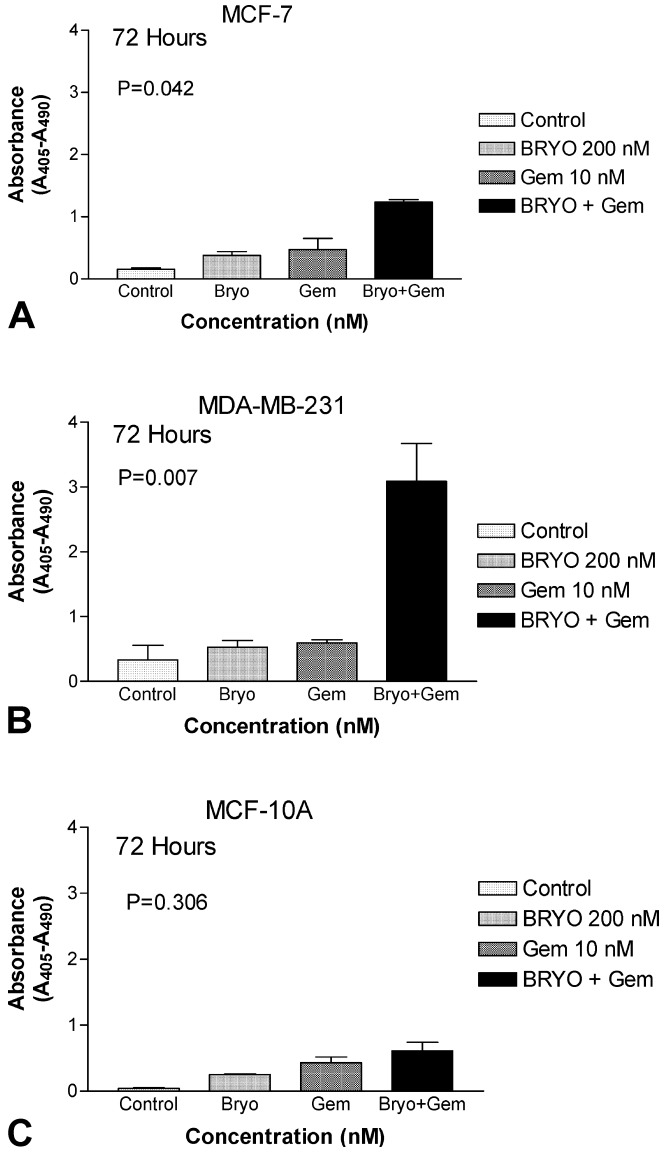


Fig. 6A–C Detection of apoptosis in exponentially growing MCF-7, MDA-MB-231 and MCF-10A human breast cells treated with BRYO, gemcitabine and the combination of the two drugs. The Cell Death Detection ELISA kit was used to measure the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Assays were performed after 24, 48, and 72 h of drug treatment. The results from the 72-h experiment repeated at least three times are shown as the means ± SD

MDA-MB-231 and MCF-10A cells was then tested. Apoptosis was determined by the Cell Death Detection ELISA^{Plus} and flow cytometry (Figs. 6 and 7, respectively). There was no significant apoptosis in cells treated with BRYO relative to DMSO-treated cells. In contrast MCF-7, MDA-MB-231 and MCF-10A cells treated with gemcitabine underwent apoptosis, which was time- and concentration-dependent. The addition of BRYO to gemcitabine increased the extent of apoptosis that was most significant ($P < 0.05$) at 72 h in the MCF-7 and MDA-MB-231 cells. MCF-10A cells treated with BRYO and gemcitabine showed no significant increase in

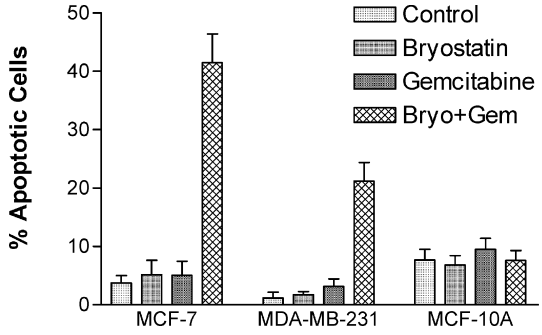


Fig. 7 Detection of apoptosis by flow cytometry in exponentially growing MCF-7, MDA-MB-231 and MCF-10A human breast cells treated with BRYO, gemcitabine and the combination of the two drugs. Both positive (heat-killed) and negative (unstained) cells were included in the assay. Samples were analyzed using a FacStar Plus flow cytometer and the data were processed by Lysis II software. The results are shown as the means ± SD

apoptosis compared to gemcitabine-treated cells. The results of flow cytometry with respect to apoptosis confirmed those obtained from ELISA assays (Fig. 7).

Expression of p53 and p21^{WAF1}, and p27 and cell cycle distribution in cells treated with BRYO and/or gemcitabine

The expression of p53, p27, and p21^{WAF1} proteins that are involved in the regulation of the cell cycle were then studied in the three cell lines treated with gemcitabine, BRYO or the combination of the two drugs (Fig. 8). A 3-day incubation with the drugs was undertaken using different concentrations. Significant induction (more than twofold) in p53, p21, and p27 was seen in the MCF-7 and MCF-10A cells treated with the combination of the two drugs. Lesser degrees of induction were seen in cells treated with either BRYO or gemcitabine. In contrast, MD-MB231 cells with the mutated p53 showed no significant induction of any of these proteins. Flow cytometric analysis of the cell cycle distribution in cells treated with BRYO, gemcitabine, or the combination relative to untreated cells was also determined (Table 1). There was no significant cell cycle effect of BRYO treatment in the cancer cells but MCF-10A cells significantly accumulated in the G_0/G_1 phase. Gemcitabine, on the other hand, resulted in reduced cell cycle progression in all three cell lines. The combination of BRYO and gemcitabine resulted in a further accumulation of MCF-10A cells in G_0/G_1 compared with cells treated with gemcitabine alone.

Expression of bcl-2, and bax in cells treated with BRYO and/or gemcitabine

The expression of bcl-2 and bax proteins that are involved in apoptosis was studied in the MCF-7, MDA-MB-231, and MCF-10A cells treated with BRYO,

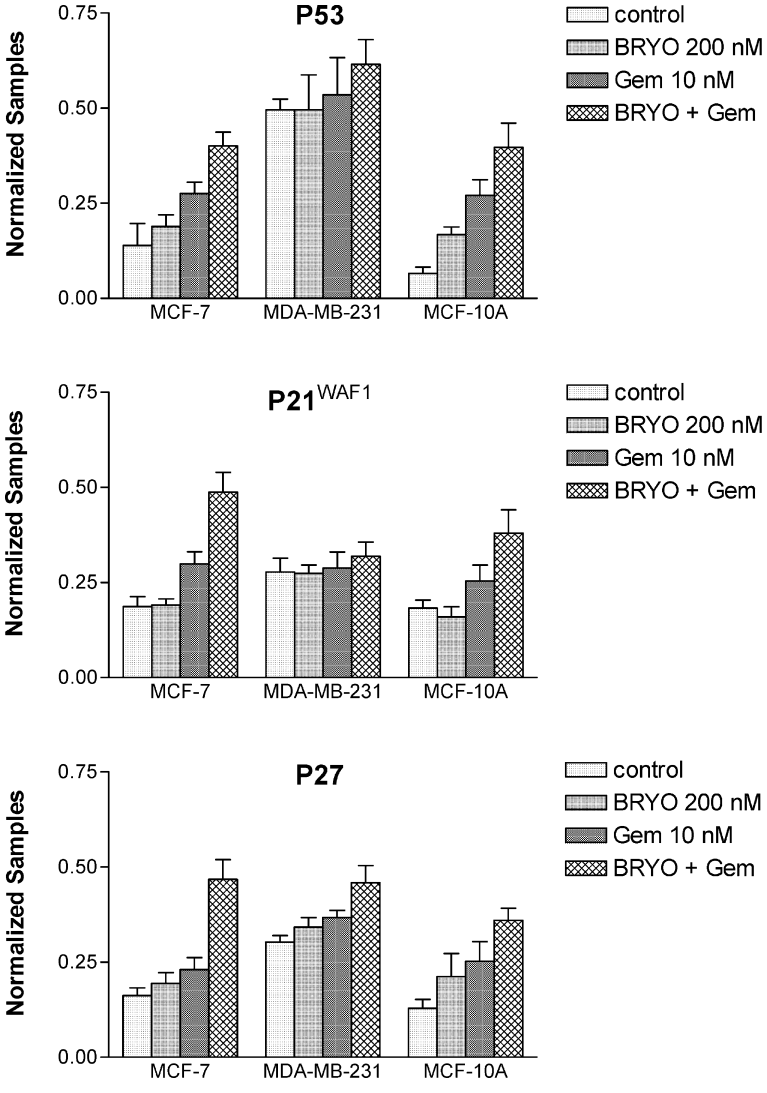


Fig. 8 The influence of BRYO on the expression of p53, $p21^{WAF1}$, and p27 proteins in MCF-7, MDA-MB-231, and MCF-10A cells. Proteins were resolved by SDS-PAGE. Primary antibodies comprised monoclonal antibodies against p53, $p21^{WAF1}$, and p27, and a polyclonal antibody against β-actin. The signal intensity of the peroxidase reaction was measured by a chemiluminescent detection system. The bidimensional optical density of proteins on the films was quantified and analyzed. The ratios p53/actin, $p21^{WAF1}$/actin, p27/actin were calculated

Table 1 Cell cycle analysis of MCF-7, MDA-MB-231 and MCF-10A cells treated with BRYO, gemcitabine and the combination

	G_0/G_1	S	G_2/M
MCF-7			
Untreated	57.8	28.5	13.8
BRYO 200 n*M*	63.8	24.7	11.5
Gemcitabine 100 n*M*	67.6	24.4	8.1
BRYO 200 n*M* + gemcitabine 100 n*M*	63.6	27.9	8.6
MDA-MB-231			
Untreated	49.0	33.0	18.0
BRYO 200 n*M*	48.6	35.7	15.7
Gemcitabine 100 n*M*	43.2	40.2	16.6
BRYO 200 n*M* + gemcitabine 100 n*M*	50.6	33.1	16.3
MCF-10A			
Untreated	49.2	22.5	28.4
BRYO 200 n*M*	60.6	11.1	28.4
Gemcitabine 100 n*M*	67.3	19.5	13.2
BRYO 200 n*M* + gemcitabine 100 n*M*	72.1	16.8	11.0

gemcitabine, or the combination of the two drugs (Fig. 9). No significant alterations in bcl-2/bax ratios were detected in cells treated with either drug alone. A significant reduction in the ratio bcl-2/bax of greater than twofold was seen in only the MCF-7 and MDA-MB-231 cells treated with the combination of the two drugs. No significant alteration in this ratio was seen in the MCF-10A cells.

Discussion

Resistance to cytotoxic therapy precludes the successful treatment of many human cancers. Signaling events in cancer cells play a crucial role in cell survival as a response to cytotoxic therapies. The PKC family of enzymes is a rational target for anticancer drug therapy because of their roles as regulators of downstream signaling molecules that determine cell response to apoptotic stimuli (Cartee and Kucera 2000). Modulation of PKC represents a rational strategy for novel therapies that may improve the efficacy of existing drugs (Basu and Lazo 1992; Jarvis and Grant 1999; Mohammad et al. 1994). Sustained activation of members of the PKC family is known to influence the growth and differenti-

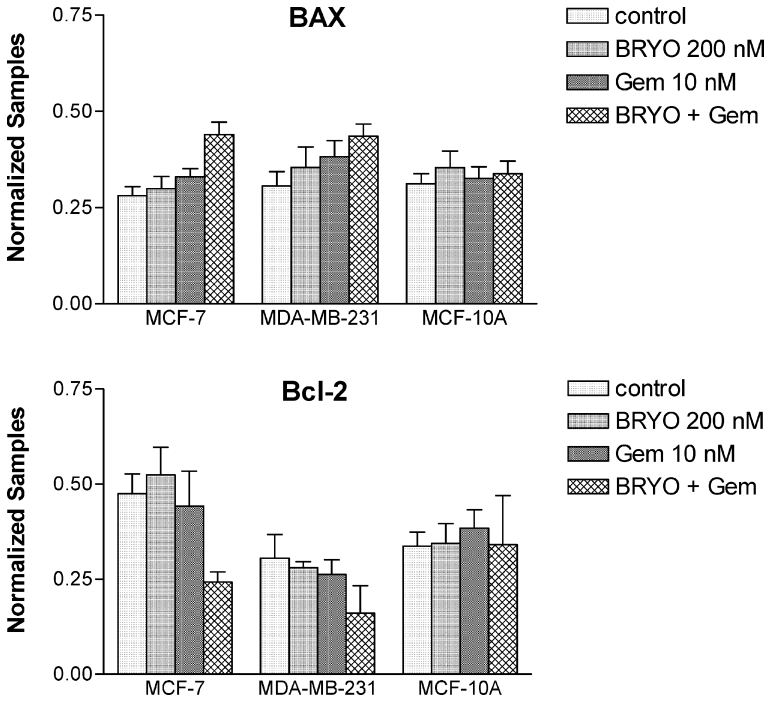


Fig. 9 The influence of BRYO and gemcitabine on the expression of bcl-2 and bax proteins in MCF-7, MDA-MB-231, and MCF-10A cells. Proteins were resolved by 10% SDS-PAGE. Primary antibodies comprised monoclonal antibodies against bcl-2 and bax, and a polyclonal antibody against β-actin. The signal intensity of the peroxidase reaction was measured by a chemiluminescent detection system. The bidimensional optical density of proteins on the films was quantified and analyzed. The ratios bcl-2/actin and bax/actin were calculated for normalization

ation of various cell types, but the specific roles of individual isoforms mediating various cellular responses have yet to be determined. Differential regulation of these enzymes by various drugs, or by the same drug in different cancer cell lines, explains the disparate results of PKC modulation with respect to proliferation and apoptosis. Results of earlier studies should be interpreted according to which PKC isoenzyme(s) were actually studied.

BRYO is a natural product that is a potent modulator of PKC (Zonder and Philip, 1999). BRYO as a single agent has shown no clinically useful antitumor activity in non-hematological malignancies (Zonder et al. 2001). Current clinical development of BRYO is focusing on its combination with cytotoxic drugs to enhance their proapoptotic activities. The major rationale for combining BRYO and gemcitabine was the known synergy between BRYO and cytosine arabinoside, an analog of gemcitabine (Jarvis et al. 1996).

BRYO is known to translocate the cytosolic PKC to the cell membrane resulting in its initial activation followed by degradation (Zonder and Philip 1999). In this study, BRYO downregulated the expression of cytosolic PKC-α, -β1, and -ϵ in MCF-7, MDA-MB-231, and MCF-10A human breast cells. The effects on the membrane-bound PKC were, however, variable. In contrast, gemcitabine had a much less-pronounced effect on PKC expression than BRYO, especially with regard to membrane PKC expression.

Under the experimental conditions of this study, BRYO alone did not significantly affect cell viability despite its marked effects on PKC expression. These finding are consistent with those of previous studies that have shown only a modest antiproliferative activity on the MCF-7 cells (Kennedy et al. 1992). BRYO had a non-significant effect on the cell cycle that was more pronounced in the MCF-10A cells. Significant induction of p53, $p21^{waf1}$ and p27 was seen only with the combination of BRYO and gemcitabine in the MCF-7 and MCF-10A cells. PKC inhibitors have been shown to induce $p21^{WAF1}$ and inhibit cyclin-dependent kinases in a manner not necessarily associated with p53 induction (Frey et al. 1997; Jeoung et al. 1995; Schwaller et al. 1997) and probably linked, at least in part, to PKC-α activation (Schwaller et al. 1997; Slosberg et al. 1999). BRYO by itself did not induce $p21^{waf1}$ in any of the cell lines tested in this study, which may be partly related to the drug concentrations used.

PKC has been shown to be directly or indirectly involved in the regulation of apoptosis induced by various cytotoxic stimuli. However, the molecular mechanisms underlying the proapoptotic activity of BRYO are poorly understood. In this study BRYO resulted in marked induction of apoptosis in the tumorigenic MCF-7 and MDA-MB-231 cells but not in the MCF-10A cells. These findings are in accord with the synergy demonstrated between BRYO and cytosine arabinoside in HL-60 cells by the enhancement of apoptosis (Jarvis et al. 1994). The bcl-2/bax ratio was downregulated only in the MCF-7 and MDA-MB-231 cells treated with the combination of BRYO and gemcitabine relative to gemcitabine-treated cells. Collectively, these results indicate potentiation between BRYO and gemcitabine, and possible selectivity to cancer cells.

The role played by PKC modulation in apoptosis induced by nucleoside analogs is poorly understood. Several molecular mechanisms leading to apoptosis may be directly or indirectly related to PKC modulation,

especially the differential modulation of the different isoforms. Possibly PKC activity is required for the repair of gemcitabine-induced DNA damage and/or DNA synthesis inhibition. Alternatively, G_1/S-arrested cells may progress through apoptosis when the PKC activity is perturbed. There is evidence to suggest that gemcitabine cytotoxicity may be influenced by PKC signaling events (Cartee and Kucera 1998). Depletion of PKC by a 24-h exposure to TPA followed by gemcitabine resulted in synergistic cytotoxicity, while coincubation of TPA with a PKC inhibitor in this regimen abrogated the synergistic response. In another study, induction of apoptosis in HL-60 leukemic cells by gemcitabine was enhanced by preincubation with the non-specific PKC inhibitor staurosporine (Bouffard and Momparler 1995). The time-course of molecular changes with respect to $p21^{WAF1}$, bcl-2 and bax expression in cells treated with BRYO (data not shown) demonstrated maximal induction of $p21^{WAF1}$ and the bax/bcl-2 ratio at 48 h with a subsequent shift to levels in untreated cells. It is likely that these changes correspond to the activation of PKC rather than its degradation following its translocation to the membrane. Regulation of phosphorylation and activity of the antiapoptotic protein bcl-2 by PKC has also been suggested in cancer cell lines (Itano et al. 1996; Ruvolo et al. 1998).

Our results show that the proapoptotic effects of BRYO were associated with differential alteration of PKC isoforms, which has been supported by other studies (Whelan and Parker 1998). There is a suggestion that PKC-βII improves cell survival with the selection of cells in the G_0/G_1 fraction (Barr et al. 1997). It is noteworthy that PKC-βII expression in the membrane was induced in the MCF-10A cells and downregulated in the MCF-7 and MDA-MB-231 cancer cells. The sustained translocation and induction of PKC-ϵ in MCF-7 cells treated with BRYO was also noteworthy and may be a major molecular target of BRYO in these cells. The growth-inhibitory effects of tamoxifen in MCF-7 cells are associated with the translocation of PKC-ϵ to the cell membrane (Cabot et al. 1997). Activation of PKC-ϵ has also been demonstrated in HT58 human lymphoblastoid cells that are growth-arrested in G_1 by phorbol myristate acetate (Mihalik et al. 1996). It has also been suggested that PKC-ϵ might be involved in cell cycle regulation independently of normal signal transduction and cyclin-controlled pathways (Han et al. 1995).

Additional studies are needed to determine the exact molecular mechanisms involved in the interaction between gemcitabine and PKC modulation in breast cancer cells. Future work must focus on the specific PKC isoenzyme(s) involved in the sensitization of cells against gemcitabine and other cytotoxic drugs. Such knowledge will greatly enhance the rational design of therapies with selective PKC modulators. Given the differential or even the opposing outcomes of PKC isoform modulation by non-isoform-selective drugs (e.g. BRYO), it is highly possible that the net cellular response(s) will be dependent on the differential modulation of the various PKC isoforms within the cell. The use of isoform-specific inhibitors of PKC isoforms (e.g., by antisense oligonucleotides) may be undertaken to determine the outcome of inhibiting specific isoforms of PKC.

The combination of gemcitabine and BRYO should be tested in the clinical setting for efficacy against human breast cancer and other gemcitabine-sensitive cancers. The lack of potentiation between the two drugs in the premalignant breast cell line also favors the testing of such a treatment in patients with breast cancer to enhance the efficacy and selectivity of cytotoxic therapy.

References

Asaoka Y, Nakamura S, Yoshida K, and Nishizuka Y (1992) Protein kinase C, calcium and phospholipid degradation. Trends Biochem Sci 17:414

Barr LF, Campbell SE, Baylin SB (1997) Protein kinase C-beta 2 inhibits cycling and decreases c-myc-induced apoptosis in small cell lung cancer cells. Cell Growth Differ 8:381

Basu A, Lazo JS (1992) Sensitization of human cervical carcinoma cells to cis-diamminedichloroplatinum (II) by bryostatin 1. Cancer Res 52:3119

Beltran PJ, Fan D, Fidler IJ, O'Brian CA (1997) Chemosensitization of cancer cells by the staurosporine derivative CGP 41251 in association with decreased P-glycoprotein phosphorylation. Biochem Pharmacol 53:245

Bouffard DY, Momparler RL (1995) Comparison of the induction of apoptosis in human leukemic cell lines by 2′,2′-difluorodeoxycytidine (gemcitabine) and cytosine arabinoside. Leuk Res 19:849

Cabot MC, Zhang Z, Cao H, Lavie Y, Giuliano AE, Han TY, Jones RC (1997) Tamoxifen activates cellular phospholipase C and D and elicits protein kinase C translocation. Int J Cancer 70:567

Carmichael J, Mitchell JB, DeGraff WG, Gamson J, Gazdar AF, Johnson BE, Glatstein E, Minna JD (1985) Chemosensitivity testing of human lung cancer cell lines using the MTT assay. Br J Cancer 57:540

Carmichael J, Possinger K, Phillip P, Beykirch M, Kerr H, Walling J, Harris AL (1995) Advanced breast cancer: a phase II trial with gemcitabine. J Clin Oncol 13:2731

Cartee L, Kucera GL (1998) Gemcitabine induces programmed cell death and activates protein kinase C in BG-1 human ovarian cancer cells. Cancer Chemother Pharmacol 41:403

Cartee L, Kucera GL (2000) Protein kinase C modulation and anticancer drug response. Cancer Invest 18:731

Chen G, Manji HK, Hawver DB, Wright CB, Potter WZ (1994) Chronic sodium valproate selectively decreases protein kinase C alpha and epsilon in vitro. J Neurochem 63:2361

Deacon EM, Pongracz J, Griffiths G, Lord JM (1997) Isoenzymes of protein kinase C: differential involvement in apoptosis and pathogenesis (review). Mol Pathol 50:124

Frey MR, Saxon ML, Zhao X, Rollins A, Evans SS, Black JD (1997) Protein kinase C isozyme-mediated cell cycle arrest involves induction of p21 (waf1/cip1) and p27 (kip1) and hypophosphorylation of the retinoblastoma protein in intestinal epithelial cells. J Biol Chem 272:9424

Gordge PC, Hulme MJ, Clegg RA, Miller WR (1996) Elevation of protein kinase A and protein kinase C activities in malignant as compared with normal human breast tissue. Eur J Cancer 32A:2120

Grant S (1997) Modulation of ara-C induced apoptosis in leukemia by the PKC activator bryostatin 1. Front Biosci 2:d242

Grant S, Jarvis WD (1996) Modulation of drug-induced apoptosis by interruption of the protein kinase C signal transduction pathway: a new therapeutic strategy. Clin Cancer Res 2:1915

Han EK, Cacace AM, Sgambato A, Weinstein IB (1995) Altered expression of cyclins and c-fos in R6 cells that overproduce PKC epsilon. Carcinogenesis 16:2423

Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Statist 6:65

Huang P, Plunkett W (1995) Induction of apoptosis by gemcitabine. Semin Oncol 22:19

Itano Y, Ito A, Uehara T, Nomura Y (1996) Regulation of Bcl-2 protein expression in human neuroblastoma SH-SY5Y cells: positive and negative effects of protein kinases C and A, respectively. J Neurochem 67:131

Jarvis WD, Grant S (1999) Protein kinase C targeting in antineoplastic treatment strategies. Invest New Drugs 17:227

Jarvis WD, Povirk LF, Turner AJ, Traylor RS, Gewirtz DA, Pettit GR, Grant S (1994) Effects of bryostatin 1 and other pharmacological activators of protein kinase C on 1-[beta-D-arabinofuranosyl]cytosine-induced apoptosis in HL-60 human promyelocytic leukemia cells. Biochem Pharmacol 47:839

Jarvis WD, Fornari FA, Traylor RS, Martin HA, Kramer LB, Erukulla RK, Bittman R, Grant S (1996) Induction of apoptosis and potentiation of ceramide-mediated cytotoxicity by sphingoid bases in human myeloid leukemia cells. J Biol Chem 271:8275

Jemal A, Thomas A, Murray T, Thun M (2002) Cancer statistics, 2002. CA Cancer J Clin 52:23

Jeoung DI, Tang B, Sonenberg M (1995) Induction of tumor suppressor p21 protein by kinase inhibitors in MCF-7 cells. Biochem Biophys Res Commun 214:361

Kennedy MJ, Prestigiacomo LJ, Tyler G, May WS, Davidson NE (1992) Differential effects of bryostatin 1 and phorbol ester on human breast cancer cell lines. Cancer Res 52:1278

Mihalik R, Farkas G, Kopper L, Benczur M, Farago A (1996) Possible involvement of protein kinase C-epsilon in phorbol ester-induced growth inhibition of human lymphoblastic cells. Int J Biochem Cell Biol 28:925

Mohammad RM, al-Katib A, Pettit GR, Sensenbrenner LL (1994) Successful treatment of human Waldenstrom's macroglobulinemia with combination biological and chemotherapy agents. Cancer Res 54:165

O'Brian C, Vogel VG, Singletary SE, Ward NE (1989) Elevated protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. Cancer Res 49:3215

Philip PA, Harris AL (1995) Potential for protein kinase C inhibitors in cancer therapy. In: Muggia FM (ed) Concepts, mechanisms, and new targets for chemotherapy (Cancer Treatment and Research, 78). Kluwer Academic Publishers, Boston, p 3

Philip PA, Rea D, Thavasu P, Carmichael J, Stuart NS, Rockett H, Talbot DC, Ganesan T, Pettit GR, Balkwill F, et al (1993) Phase I study of bryostatin 1: assessment of interleukin 6 and tumor necrosis factor alpha induction in vivo. The Cancer Research Campaign Phase I Committee. J Natl Cancer Inst 85:1812

Prendiville J, Crowther D, Thatcher N, Woll PJ, Fox BW, McGown A, Testa N, Stern P, McDermott R, Potter M, et al (1993) A phase I study of intravenous bryostatin 1 in patients with advanced cancer. Br J Cancer 68:418

Ruvolo PP, Deng X, Carr BK, May WS (1998) A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis. J Biol Chem 273:25436

Schwaller J, Peters UR, Pabst T, Niklaus G, Macfarlane DE, Fey MF, Tobler A (1997) Up-regulation of p21WAF1 expression in myeloid cells is activated by the protein kinase C pathway. Br J Cancer 76:1554

Slosberg ED, Klein MG, Yao Y, Han EKH, Schieren I, Weinstein IB (1999) The alpha isoform of protein kinase C mediates phorbol ester-induced growth inhibition and p21(cip1) induction in HC11 mammary epithelial cells. Oncogene 18:6658

Stabel S (1994) Protein kinase C—an enzyme and its relatives. Semin Cancer Biol 5:277

Whelan RD, Parker PJ (1998) Loss of protein kinase C function induces apoptotic response. Oncogene 16:1934

Zonder J, Philip PA (1999) Pharmacology and clinical experience with bryostatin 1: a novel anticancer drug. Expert Opin Invest Drugs. 8:2189

Zonder J, Shields AF, Zalupski M, Chaplen R, Heilbrun LK, Philip PA (2001) A phase II trial of bryostatin 1 in the treatment of metastatic colorectal cancer. Clin Cancer Res 7:38