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# Apoptosis of MCF7 Cells Treated with PKC Inhibitors and Daunorubicin

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**Abstract**

**The present study was performed to observe the role of protein kinase C (PKC) inhibitors (H-7, staurosporine) and daunorubicin in the cell death process of MCF7 cells; and examined whether or not the type of induced cell death was apoptosis. The usefulness of the combined therapy of PKC inhibitors and daunorubicin to improve the adverse effect of daunorubicin was also investigated. Cell death was induced by treatment with PKC inhibitors or daunorubicin. Characteristic morphologic features of cell shrinkage, chromatin condensation, and cytoplasmic vacuolization were observed. These treatments also stimulated the cleavage of poly-(ADP-ribose) polymerase (PARP), an early event in apoptosis. With slight differences in the percentage of apoptosis-induced cells, staurosporine, H-7 and daunorubicin effectively induced apoptosis in MCF7 cells. Furthermore, combined treatment of PKC inhibitors and daunorubicin significantly drove the cells into an apoptotic state. Hence, our results revealed the possible therapeutic value of combined therapy for the prevention of drug resistance and adverse side effects.**

**Key words:** apoptosis, protein kinase C, MCF7 cells, daunorubicin

## INTRODUCTION

The regulation of cell proliferation is paramount for the integrity of the individual, and the unregulated proliferation of cell can be catastrophic for the individual, resulting in cancer. Cell death is tied to the same signals that activate cell proliferation. Thus, there is an intimate association between the regulation of cell proliferation and that of cell death, which controls the growth of tissues and is responsible for preventing the onset of neoplasm. Cell death can play a central role in the treatment of neoplasm as well as development of tumor. Apoptosis, a physiologic form of cell death, is induced in cancer cells by certain treatment modalities such as radiotherapy and cytotoxic chemotherapy (1,2).

The anthracycline antibiotics (doxorubicin and daunorubicin) are widely used in the treatment of a variety of neoplastic diseases, and may be one of the most important categories of antitumor drugs currently available. The success of daunorubicin in patients with neoplasms is limited by the emergence of resistant cell populations and by a dose-limiting cardiotoxicity. Either new strategies or new drugs are needed to overcome these limitations.

PKC is a ubiquitous serine/threonine-protein kinase that is thought to play a crucial role in growth regulation, differentiation, and malignant transformation. It has been reported that PKC activators or inhibitors can cause apoptosis, depending on cell type. There have been a few reports describing the role of PKC inhibitors and daunorubicin on MCF7 breast cancer cells (3-5). Accordingly, in this study, we further characterized the effect of PKC inhibitors and daunorubicin on the cell death process in MCF7 cell and investigated whether or not the type of induced cell death was apoptosis. The possibility of the combined therapy of PKC inhibitors and daunorubicin to circumvent the defects of daunorubicin, drug resistance and cardiotoxicity, was also examined.

## MATERIALS AND METHODS

### Cell culture and preparation of cells

MCF7 cells, a human breast cancer cell line, were provided by the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL). Cells were maintained in an atmosphere of 5% $CO_2$ in air. Cells were treated with 200 nM staurosporine, 30 μM 1-(5-isoquinolinylsulfonyol)-2-methylpiperazine (H-7) and 0.3 μM daunorubicin for 2.5 h. Otherwise cells were incubated with staurosporine and daunorubicin or with H-7 and daunorubicin for 2.5 h. Cells grown in the presence or absence of FBS without treatment with any reagent were used as control groups. For simplicity of description, each group will

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be abbreviated as follows: C, Control group; R, Control group with serum deprivation; St, group incubated with 200 nM staurosporine; H-7, group treated with 30 μM H-7; Da, group incubated with 0.3 μM daunorubicin; SD, group treated with 200 nM staurosporine and 30 μM daunorubicin; HD, group incubated with 30 μM H-7 and 0.3 μM daunorubicin.

**Proportions of apoptotic cells and morphological analysis**

The cells were seeded and grown in Lab-Tek chamber slides for 24 h and then treated with the above-mentioned chemicals. They were stained with a 2 μL mixture of 270 μM acridine orange and 254 μM ethidium bromide (1 : 1) for the discrimination of apoptotic cells. The cells were examined under a fluorescence microscope (Olympus, Japan). It has been established that cells are green when they are not apoptotic and brownish or reddish colored if cell death has occurred. When a cell had lost any portion of its green color and/or exhibited red color, it was considered to be an apoptotic change, in the present study. The number of apoptotic cells was counted with a hemocytometer under a fluorescence microscope. The morphologic changes of the cell were also examined.

**Laser cytometry**

Cytometry was used to invetigate which parts of the cells were affected by apoptosis. Cells were cultured in 35 mm ACAS dishes (Mat-Tek, USA) and treated with chemicals as described above. They were then stained with a 2 μL mixture of 270 μM acridine orange and 254 μM ethidium bromide (1 : 1). The images of the cells were displayed on the monitor and photographed using a dual image program at the resolution of 1024×1024 pixels. Laser cytometry was established with the following parameters: 10% transmission, 100 μM pinhole, 589 nm emission filter, 600 V photomultiplier voltage, and 488 nm excitation wave length. The cell images were measured exactly at single optical sites and analyzed by a color video copy processor (CP2000, Mitsubishi Co., Japan).

**Western blotting**

For protein analysis, cells were harvested, washed in PBS, and lysed in RIPA buffer (10 mM Tris/HCL, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 1 mM EGTA, 250 μg/mL leupeptin, 0.1% PMSF, 1 mM benzamidine, 1% aprotinin) on ice for 30 min. Lysates were clarified by centrifugation at 15,000×g at 4°C for 20 min. The protein concentration was determined by Bradford assay. Protein samples (30 μg) were electrophoresed through a 7.5% polyacrylamide gel and transferred to Immun-Blot PVDF membrane (Bio-Rad). The membrane was blocked with blocking solution (5% non-fat dry milk, 0.05% Tween 20 in Tris-NaCl pH 7.8) for 1 h at room temperature. The membrane was then incubated with a polyclonal PARP antibody (Pharmingen Co.), diluted 1/1,000 in a solution containing 5% non-fat dry milk and 0.05% Tween 20 in Tris-NaCl pH 7.8, overnight at 4°C. After three washes using 20 mM Tris buffer (pH 7.8), the membrane was incubated with secondary biotinylated anti-rabbit IgG antibody at a dilution of 1 : 1000 (Sigma) at room temperature for 1 h. After three final washes, avidin-biotin complex solution was added to the membrane and incubated for 1 h. The membrane was rinsed three times, and the bands were visualized by adding 3,3-idaminobenzidine tetrahydrochloride (Dako Corp.).

**Analysis of DNA replication**

Five million cells were centrifuged at 1000×g for 5 min. The pellet was lysed in 50 μL of a buffer consisting of 0.5% Trition X-100, 5 mM Tris and 20 mM EDTA on ice for 20 min. Thereafter, 10 μL of a solution of 20 mg/mL proteinase K was added. The samples were left at 55°C overnight; after which a 10 μL solution of 0.5 mg/mL RNase A was added and the samples kept at 37°C for 1 h. After centrifugation at 15,000×g for 10 min, the lysates were extracted three times with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The supernatant was adjusted to 0.3 M sodium acetate, and DNA was precipitated with two volumes of cold ethanol. After overnight incubation at -20°C, DNA was collected by centrifugation (15,000×g, 4°C) for 10 min, washed in 500 μL of 70% ethanol by centrifugation, and air-dried. Extracted DNA was treated at 37°C for 2 h with a mixture of reagents (40 μL) containing: 40 mM creatine phosphate-di-Tris salt (pH 7.7), 1 μg creatine kinase, 7 mM $MgCl_2$, 5 μg bovine serum albumin, 0.5 mM DTT, 4 mM ATP (pH 7.5), 334 μM NTPs (except TTP), 100 μM dNTP (except dTTP), 25 μM [$^3$H]dTTP (1000 cpm/pmol), 0.5 μg SV 40Tag, and 0.25 μg SV40 origin-containing DNA (pUC-ori$^+$). Amounts of replication proteins and extracts were measured using a scintillation counter (Wallac, Finland).

## RESULTS

Apoptosis is a physiologic form of cell death involved in the regulation of cell number in several physiologic and pathologic conditions, including: morphogenesis, thymic selection, and leukocyte senescence (6). It is characterized by distinct morphologic and biochemical features. Morphologic features of apoptosis include chromatin condensation, cell shrinkage, nuclear fragmentation and formation of membrane bound "apoptotic bodies". These findings

are confined to nuclear changes in apoptotic cells; cytoplasmic change in apoptotic cell have not been reported.

**Proportions of apoptotic cells and morphological analysis**

H-7 induced cell shrinkage, decreased population, cytoplasmic vacuolization and nuclear condensation in the MCF7 cells. Unlike staurosporine, H-7 caused apoptosis in the nucleus reflecting DNA damage because H-7 functions as a DNA damaging agent as well as a PKC inhibitor. There were no apparent changes in the cells of the control groups. In the St, H-7 and Da groups, 28.03%, 54.60% and 61.42% of cells exhibited apoptotic change, respectively. Among them daunorubicin was the most potent agent in inducing apoptosis. Co-treatment of daunorubicin and PKC inhibitors was more cytotoxic than treatment with any single agent in driving the cells to an apoptotic state (Fig. 1). It is thought that the efficacy was due to the combined effects of inhibition of cell growth and cytotoxicity of the chemicals.

We were unable to see morphological apoptotic features such as cell shrinkage, cytoplasmic blebbing, or chromatin condensation in the cells of the C and R groups (Fig. 2-a, b). In the cells of the St group, apoptotic change was observed in the cytoplasm rather than in the nucleus. The color of the cytoplasm was changed to red and cytoplasm vacuolization was readily detected (Fig. 2-c).

In the H-7 treated group, morphologic changes of the nucleus, mainly nuclear condensation, were readily detected (Fig. 2-d). In the cells treated with daunorubicin, which has an inhibitory effect on the development and differentiation of human breast cancer cells, the cytoplasm was perfectly stained in red and showed vacuolar change. The red color was also apparent in the nucleolus within the nucleus (Fig. 2-e).

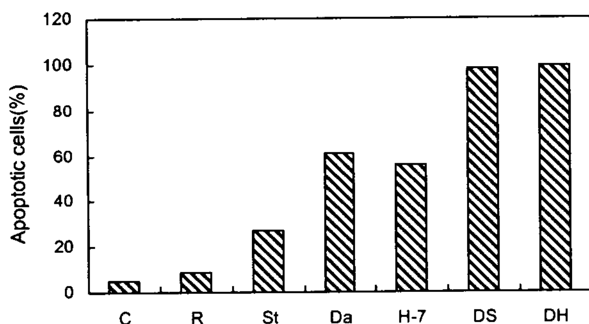


**Fig. 1.** Cell growth of apoptotic cells in the MCF-7 cells treated with PKC inhibitors and/or daunorubicin. The treated cells were cultured in Lab-Tek chamber slides, and stained with acridine orange and 254 μM ethidium bromide (1 : 1). C: Control (with serum), R: Control (without serum), St: 200 nM Staurosporine, Da: 0.3 μM Daunorubicin, H-7: 30 μM 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, DS : 30 μM Daunorubicin/200 nM Staurosporine, DH : 0.3 μM Daunorubicin/ 30 μM H-7.

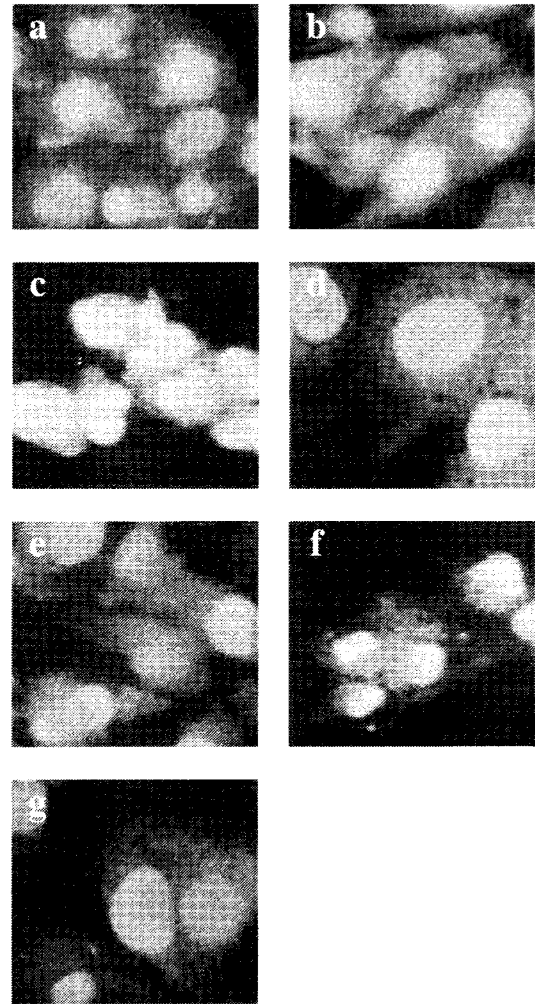


**Fig. 2.** Apoptotic cell death in treatment of PKC inhibitors and daunorubicin to MCF-7 cells for 2.5 h using fluorescense microscopy. Each cell group was stained with 270 μM acridine orange and 254 μM ethidium bromide (1 : 1). Apoptotic cells were observed by reflected fluorescence microscopy (Olympus, Japan). a: control (w/serum), b: control (w/o serum), c: staurosporine, d: daunorubicin, e: H-7, f: daunorubicin/staurosporine, g: daunorubicin/H-7.

In the SD and DH groups, loss of the cytoplasm and vacuolization were observed to some degree. The blebbing of plasma membrane and nuclear envelope appeared as well, indicating the complete induction of apoptosis in the nucleus. There was no significant difference of morphologic features between SD and DH groups (Fig. 2-f, g).

**Laser cytometry**

The authors observed the location of apoptotic change occurring in the cells by using the ACAS. In the St group, apoptotic features mainly appeared in the cytoplasm (Fig. 3-c). H-7 caused damage to the nucleus rather than to the cytoplasm (Fig. 3-d). The Da group showed apoptotic features throughout the whole area of the cells, suggesting

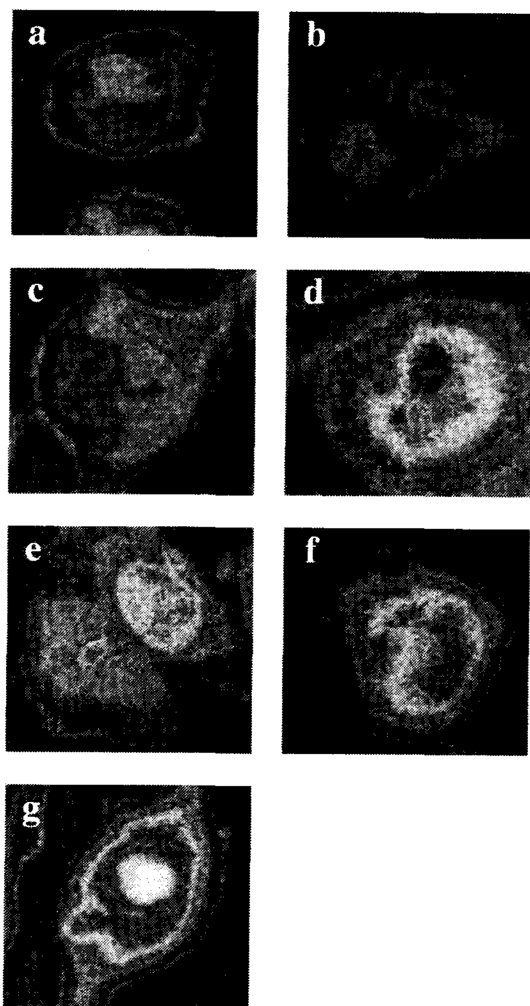


**Fig. 3.** Apoptotic cell death in treatment of PKC inhibitors and daundrubicin to MCF-7 cells for 2.5 h by using laser cytometry. Laser cytometry was established with the parameter of 10% transmission, 100 μM pinhole, 589 nm emission filter, 600 V photomultiplier voltage, and 488 nm excitation wave length. The cell images were measured exactly at single optical sites and analyzed by a color video copy processor (CP2000, Mitsubishi Co., Japan). a: control (with serum), b: control (without serum), c: staurosporine, d: daunorubicin, e: H-7, f: daunorubicin/staurosporine, g: daunorubicin/H-7.

that both the nucleus and the cytoplasm were damaged by daunorubicin (Fig. 3-e). Most of the cells in the SD and DH groups exhibited red color in both the cytoplasm and the nucleus. Although green color was observed; it was restricted to a small part of the nucleus, indicating that complete cell death had occurred (Fig. 3-f, g).

**Analysis of western blot**

We examined cell lysates of MCF7 cells for cleavage of PARP, which is known to reflect apoptotic cell death. The cleavage of PARP could be readily assessed by the appearance of corresponding fragments in cell lysates of drug-treated groups, whereas no fragments were detected in the C and R groups (Fig. 4). Based on these finding, the authors confirmed that the morphologic change and/ or loss of cell viability is caused by apoptosis.

**Analysis of DNA replication**

A DNA replication assay was used in order to determine the correlation between DNA fragmentation and the cell cycle in the cells undergoing apoptotis. The co-treatments of a PKC inhibitor with daunorubicin was most effective in inhibiting DNA replication recovery by causing cell death and concurrently blocking the cell cycle. Ten point one pM of DNA replication was measured in the 1.6650 mg/mL extract of the C group, but that value was reduced to 5.8 pM in the R group. The St group showed 6.0 pM of DNA replication, which was higher than that of the R group. It is thought that staurosporine acts only as a PKC inhibitor, whereas H-7 has a direct DNA-damaging effect, inhibiting DNA replication. Although the H-7 and Da groups had similar cell death ratios, daunorubicin affected DNA replication more efficiently than treatments with any single agent (Fig. 5).

## DISCUSSION

Staurosporine, which has a high dose-dependency, induced apoptosis at concentrations of more than 1 μM as previously described (7). Previous studies have mainly focused on apoptosis in the nucleus, and revealed that the apoptotic change was due to accumulation of p53 protein in the nucleus. In the present study, low concentrations of staurosporine (200 nM) induced death process in the cytoplasm, whereas it did not promote apoptosis in the nucleus. This means that staurosporine can induce apoptosis, not by DNA damage and resultant p53 accumulation, but by some other mechanism. These results suggest that cytoplasmic apoptosis precedes nuclear apoptosis at low concentrations of staurosporine. H-7 can also increase

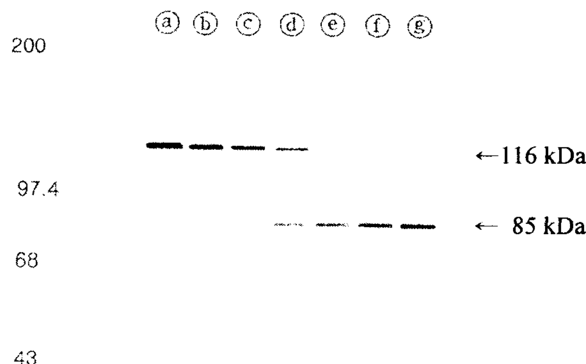


**Fig. 4.** Cleavage of PARP in MCF-7 cell lysates by treatment of PKC inhibitors and daunorubicin for 2.5 h viewed in a western blotting. a: control (w/serum), b: control (w/o serum), c: staurosporine, d: daunorubicin, e: H-7, f: daunorubicin/staurosporine, g: daunorubicin/H-7.

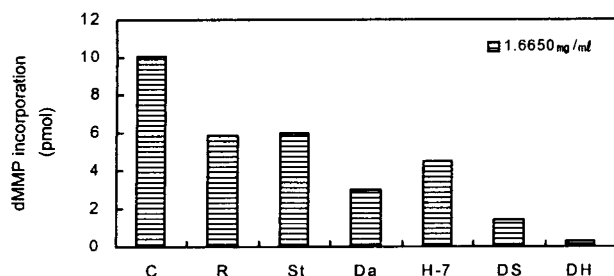


**Fig. 5.** The inhibitory effects of PKC inhibitors and daunorubicin in SV40 monopolymerase replication *in vitro*. C: control (w/serum), R: control (w/o serum), St: staurosporine, Da: daunorubicin, H-7: 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, DS: daunorubicin/staurosporine, DH: daunorubicin/ H-7.

the stability of p53, causing the accumulation of p53 in the nucleus. As a result, it can arrest the cell cycle, and then cause apoptosis (8). Further study to define the characteristics of cytoplasmic apoptosis and their meaning or effect is needed, and the role of cytoplasmic apoptosis at high concentration of staurosporine should be clarified in the future.

Daunorubicin is known to be highly cytotoxic to cancer cells because it stays in the nucleus and blocks the growth of cancer cells. Daunorubicin induces the synthesis of ceramide from sphingomyelin or sphingoglycolipids by sphingomyelinase. This ceramide has a role as a second messenger related to the inhibition of cell proliferation and differentiation, and the induction of apoptosis. It also transfers the apoptotic signals to the IL-1β converting enzyme (ICE) family cascade. After the activation of the ICE family, the enzymes induce apoptosis (9-13). Daunorubicn generally causes a reduction in the cell population, cell shrinkage, breakdown of membrane integrity and DNA fragmentation (13,14). As shown in the St and H-7 groups, cells treated with daunorubicin also exhibit apoptotic features such as cell shrinkage, reduction in the cell population, and nuclear condensation; causing damage throughout the cell. We confirmed that daunorubicin has the ability to kill the cancer cells, but its use is limited due to appearance of drug resistant cells and cardiotoxicity.

The co-treatments of a PKC inhibitor and daunorubicin showed an increased cytotoxicity, compared with the treatments of each chemical alone. The co-treatment of H-7 and daunorubicin exerted the most powerful cytotoxic effect. It is likely that co-treatment can promote apoptosis in the nucleus and in the cytoplasm at the same time. Our data indicate that combined treatment of PKC inhibitors and daunorubicin are effective treatment protocols, overcoming the emergence of drug resistance and/or cardiotoxicity.

## ACKNOWLEDGEMENTS

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