N-(p-coumaroyl) serotonin induces cell cycle arrest and apoptosis in breast cancer cells

Michail Mitsis[1], George A. Alexiou[2], Evrysthenis Vartholomatos[2], Georgios Markopoulos[3], Diamanto Lazari[4], Entela Hodaj[4,5], Dimitrios Nastos[1], Panagiota Zagorianakou[2], Vasiliki Galani[6], Athanasios P. Kyritsis[2]

[1]Department of Surgery, Ioannina University Hospital & University of Ioannina, Ioannina, Greece; [2]Neurosurgical Institute, Medical School, University of Ioannina, Ioannina, Greece; [3]Laboratory of Biology, School of Medicine University of Ioannina, and Division of Biomedical Research IMBB-FORTH University Campus, Ioannina, Greece; [4]Laboratory of Pharmacognosy, Division of Pharmacognosy-Pharmacology, School of Pharmacy, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece; [5]Department of Industrial Chemistry, Faculty of Natural Sciences, University of Tirana, Tirana, Albania; [6]Department of Anatomy-Histology-Embryology, Medical School, University of Ioannina, Ioannina, Greece

Summary

Purpose: *Breast cancer is the most commonly diagnosed malignancy among women. Breast cancer cells may develop resistance to current chemotherapy, thus new chemotherapeutic agents are urgently needed.*

Methods: *A major number of drugs with anticancer activity have been isolated from plants. Herewith, we investigated for the first time the effect of N-(p-coumaroyl) serotonin (CS), isolated from Centaurea seed on a drug-resistant breast carcinoma (MCF-7) cells. Viability and proliferation of the cells were examined with trypan blue exclusion assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Caspace-8, cell cycle, and CD24/CD44/CD56/CD58/CD71/CD15 expression were tested with flow cytometry.*

Results: *Treatment with CS significantly reduced cell viability. Induction of cell death and cell cycle arrest was confirmed with flow cytometry. After treatment with CS, there was a dose-dependent decrease in CD24/CD44/CD58/CD71 expression, whereas there was no change in CD56 and CD15 expression.*

Conclusion: *The treatment of breast cancer cells with CS may represent a novel therapeutic strategy and requires further investigation.*

Key words: *apoptosis, breast cancer, cell cycle, N-(p-coumaroyl) serotonin*

Introduction

Breast cancer is the most commonly diagnosed malignancy among American and Northwestern European women. Nearly one-third of the women with breast cancer will present with metastases and succumb to the disease [1]. Breast cancer cells may develop resistance to current chemotherapy, thus new chemotherapeutic agents are urgently needed. A major number of drugs with anticancer activity has been isolated from plants [2,3]. We have recently showed that N-(p-coumaroyl) serotonin (CS), a serotonin conjugate found in plants, including *Centaurea* seed and safflower, holds antiglioma properties [4]. Furthermore, CS has been previously demonstrated antioxidative, fibroblast proliferation and cardioprotective activity [5-8]. In the present study we investigated for the first time the effect of CS on a drug-resistant breast carcinoma (MCF-7) cells.

Methods

Cell lines and treatment conditions

The human breast cancer cell line MCF7 was obtained from Dr Koletas (Department of Biology, University Hospital of Ioannina). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Grand Island, NY) that was supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin (Gibco BRL) and grown at 37°C in a 5% CO_2 atmosphere. CS was dissolved in DMSO to obtain stock solutions. Cultures of malignant breast cancer cells were treated with increasing concentrations of CS.

Viability assay

Cultures of human breast cancer cells were treated with CS at concentrations of 100, 200, 300 and 400 µM. Cell viability was examined by trypan blue exclusion assay and MTT. Three experiments were performed for each assay and the results are represented as the mean of different experiments. Cell cultures were observed every day by light microscopic observation and viability tests were performed at 24 and 48 hrs after treatment with CS.

Flow cytometric analysis of DNA cell cycle

Cells were treated with CS at concentrations of 100, 200 and 300 and 400 µM and they were also left untreated as a negative control. All samples were run in triplicate in at least three independent experiments. Flow cytometric analysis for propidium iodide (PI) was carried out at 72 hrs. For analyzing DNA cell cycle, cells were trypsinized, centrifuged, washed with buffer PBS and incubated with PI-working solution (50 µg/mL PI, 20 mg/mL RNase A and 0.1% Triton X-100) for 20 min at 37°C in the dark. The PI fluorescence of 10,000 individual nuclei was measured using a flow cytometer (FACScalibur, Becton Dickinson San Jose, California, USA). Cell Quest software program (BD Biosciences) was used and the fractions of the cells in G0/G1, S, G2/M and sub-G0/G1 phase were analyzed and calculated for each histogram as the mean peak fluorescence intensity.

Flow cytometric analysis of Caspase-8 activity

Caspase-8 activity was performed with Fluorescein Active Caspase-8 Staining Kit (Abnova, Taiwan) as previously described [9]. Cells were cultured in 24-well plates and treated as described at viability assay. The cells were then trypsinized and 1 µl of FITC-IETD-FMK was added into each tube. Each tube was incubated for 0.5-1 hr at 37°C incubator with 5% CO_2. After centrifuge at 3000 rpm (5 min) cells were resuspended in 0.5 ml of Wash Buffer and centrifuged again. Flow cytometry was used for quantification of fluorescent cells as described above.

Flow cytometric analysis of mitochondrial membrane potential (ΔΨn)

Mitochondrial membrane potential was analysed using BD mitoscreen Mitochondrial membrane Potential Detection kit, according to manufacturer's protocol. Briefly, cells were trypsinised, incubated for 15 min with JC-1 solution, washed 3 times and analysed with flow cytometry. Green and red fluorescence from 10,000 cells was analysed to differentiate between cells with intact mitochondria (high membrane potential) and cells undergoing apoptosis (significantly lower membrane potential), using the appropriate gates.

CD24/CD44/CD15/CD56/CD58/CD71 analysis by flow cytometry

Cells were treated with CS at concentrations of 100, 200, 300 and 400 µM. Untreated cells were used as negative control. Cells were dissociated by trypsinization, washed twice with PBS and, in order to block Fc receptors, they were incubated with 10% human serum for 20 min on ice. FITC Mouse Anti-Human CD24 (ML5), PE Mouse Anti-Human CD24 (ML5), FITC Mouse Anti-Human CD44 (Leu-44), FITC Mouse Anti-Human CD56 (NCAM16.2), FITC Mouse Anti-Human CD58 [1C3 (AICD58.6)], FITC Mouse Anti-Human, CD15 (W6D3), FITC Mouse Anti-Human CD71 (M-A712) and PE Mouse Anti-Human CD71 (M-A712) antibodies (all from BD Pharmingen) were added and incubated for 20 min on ice in the dark. The cells were washed twice with PBS, finally resuspended in PBS and analysed by a flow cytometer (FACScalibur, Becton Dickinson San Jose, California, USA). Analysis was performed using Cell Quest software program (BD Biosciences). The results were determined for each histogram as the geometric mean peak fluorescence intensity. Quantitative measurement of bound anti-CD24, anti-CD44, anti-CD15, anti-CD56, anti-CD58 and anti-CD71 antibodies was achieved using the flow cytometry based QIFIKIT® assay (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. A standard curve comparing the geometric mean of fluorescence to known FITC content of the Quantibrite beads was constructed using QuantiCALC software (BD Biosciences).

Statistics

The data were expressed as mean±SD. Mann-Whitney U test was used to determine the significance of differences between experimental conditions. Differences were considered significant at p values <0.05.

Results

IC_{50} and sensitivity to CS of MCF-7 cells

MTT assay was used to calculate the cytotoxicity effects on MCF-7 cells, after CS treatment. We found that CS induced cytotoxicity, 48 hrs post treatment. IC_{50} values based on MTT assay were 349 µM during 48-hr treatment. To examine the cytotoxic effects of CS we also utilized the trypan blue exclusion test. We determined the cell survival rate, which was defined as the number of living cells at 48 hrs after exposure to escalating dos-

es of CS compared with the number of live control cells. The number of MCF7 cells significantly decreased with escalating doses of CS (Figures 1,2).

CS induced cell cycle arrest

To examine the cell cycle events underlying these observed growth inhibitory effects, we assessed the effect of CS on cell cycle progression of the MCF-7 cells. These cells were exposed to

increasing concentration of CS (100, 200, 300 and 4200 µM) for 48 hrs. CS produced an S-phase arrest in a dose-dependent manner in MCF-7 cells (Figure 3).

CS increased the activation of Caspase-8 in MCF-7 cells

Next, we evaluated the effect of CS in caspase-8 activation. 10^4 MCF-7 cells were seeded and 24 hrs later the cells were exposed to CS at 200 and 400 µM. The results showed that CS produced significant higher activity of caspase-8 compared to control (Figure 4).

CS induced mitochondrial-independent apoptosis in MCF7 cells

To further study whether the apoptosis induced by CS was associated with mitochondrial membrane depolarization, untreated MCF7 cells or CS-treated cells (200 and 400 µm for 24 hrs) were stained with JC-1 and analysed by flow cytometry. JC-1 emmits fluorescence in both the FL-2 and FL-1 channels and considered to correspond
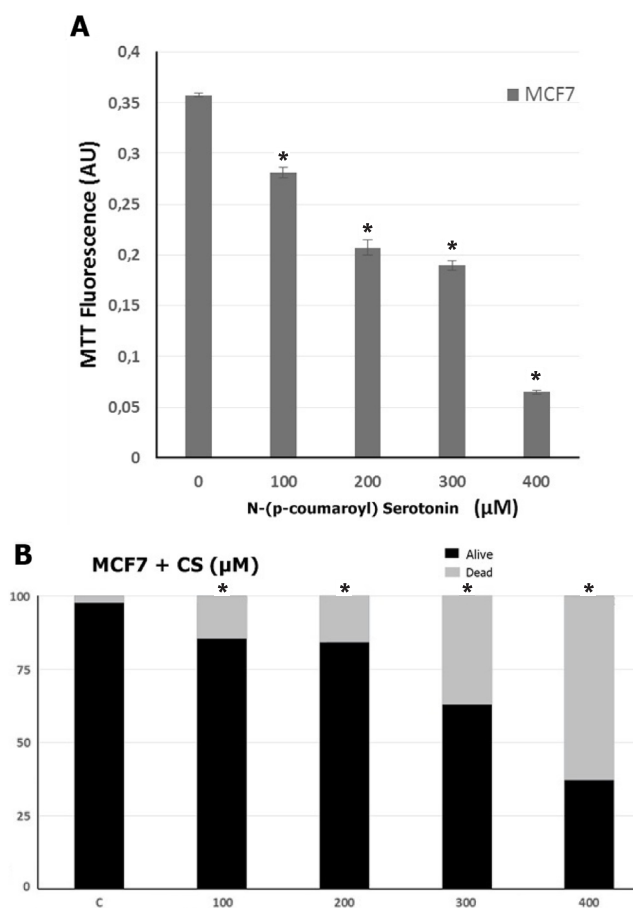

Figure 1. A: Results of the MTT assay on MCF7 after CS treatment. **B:** Viability of breast cancer cells following CS treatment. Cell viability was assessed by the trypan blue exclusion test in MCF7 cells. 10^4 breast cancer cells were seeded in a 24-well-plate and 24 hrs after seeding cells were treated with escalating concentration of CS (100, 200, 300 and 400 µM). Viability tests were performed on day 2. Values are normalized to nontreated cells. *p<0.05 vs control.
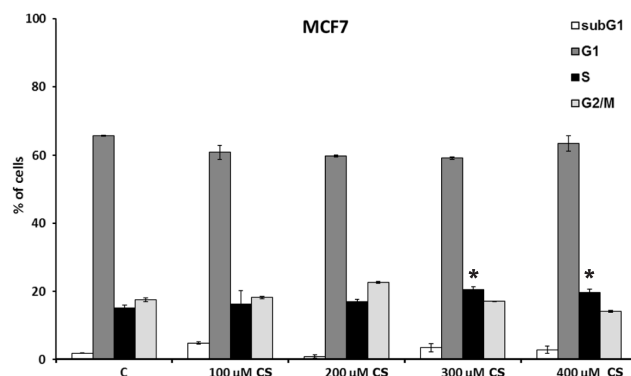

Figure 3. The effect of CS in cell cycle. MCF-7 cells (10^5) were seeded in 24-well plate and after 24 hrs were exposed to escalating concentration of CS (100, 200, 300 and 400 µM) for another 48 hrs. At 48 hrs, cells were stained by propidium iodide and the DNA content was evaluated by flow cytometry. CS produced S-phase cell cycle arrest in a dose-dependent manner. An increase in the sub-G0/G1 phase fraction (M4) was also observed suggesting apoptosis. *p<0.05 vs control.
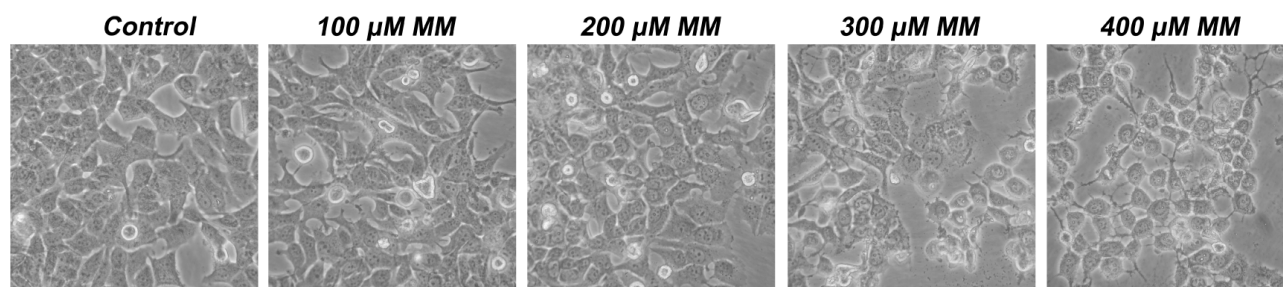

Figure 2. The effects of escalating concentration of CS in MCF-7 cells (x20). Treatment with 100-400 µM of CS led to gradual cell reduction (partial reduction of cell confluence) and also to cytotoxic effects (round cells more prominent in higher doses).
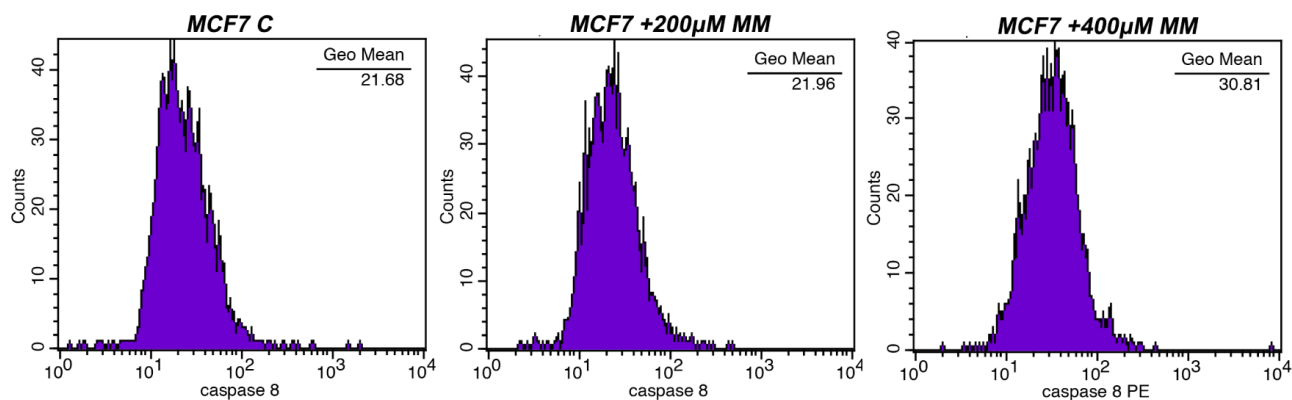
Figure 4. Representative histograms showing the activation of caspase-8. MCF-7 cells (10^5) were seeded in 24-well plate and after 24 hrs were exposed to CS (200 and 400 μM) for another 24 hrs. A significant increase in the activation of caspase-8 was observed after treatment with CS. C: control, PE: phycoerythrin.
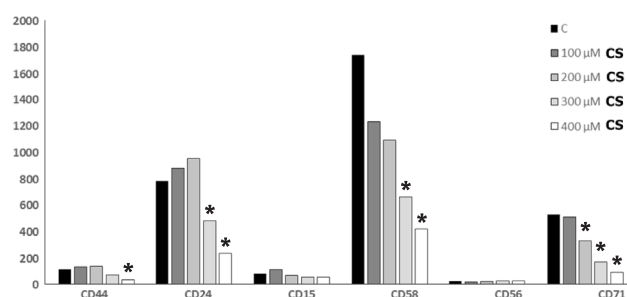

Figure 5. Flow cytometry analysis for the expression of CD24/CD44/CD56/CD58/CD15/CD71 in MCF-7 cells. MCF-7 cells (10^5) were seeded in 24-well plate and after 24 hrs were exposed to escalating concentrations of CS (100, 200, 300 and 400 μM) for another 48 hrs. There was a dose-dependent decrease in CD24, CD44, CD58 and CD71 expression. No significant change was noted for the CD56 and CD15 expression after CS treatment. *p<0.05 vs control.

to mitochondria with a polarized Δψ. JC-1 that fluoresces in the FL-1 channel and lacks fluorescence in the FL-2 channel is considered to correspond to mitochondria with a depolarized Δψ. After CS treatment there was no significant increase in the number of cells with lowered red fluorescence, indicative of no change in the Δψ.

CS decreased the expression of CD24, CD44, CD71 and CD58 expression

We then analysed the expression of CD24, CD44, CD58 and CD71 by flow cytometry. After CS treatment there was a dose-dependent significant decrease in the number of CD24/CD44/CD58/CD71 molecules/cell in MCF7 cells (Figure 5).

CS did not affect significantly CD56 and CD15 expression

We then analysed the expression of CD56 and CD15 by flow cytometry. No significant alteration in the CD56 and CD15 expression was found after CS treatment in MCF7 cells (Figure 5).

Discussion

The present study showed that CS may be a potential new treatment for breast cancer lines *in vitro*. CS induced suppression of breast cancer cell growth, S-phase cell cycle arrest and apoptosis in MCF7 cell line, also associated with activation of caspase-8 and mitochondrial-independent. CS also produced decreased CD/44/CD24/CD56/CD58/CD15 and CD71 expression.

In a previous study on glioma cell lines we have shown that CS significantly reduced cell viability in U251MG and T98G cells [4]. CS also led to induction of cell death via apoptosis and cell cycle arrest at G2/M and S-phase. After treatment with CS there was a dose-dependent increase in CD15 and CD71 expression, whereas there was no change in CD24/CD44/CD56 expression in both cell lines. Furthermore, in order to evaluate the possible toxicity CS, we used zebrafish embryos and we found no toxicity even at a concentration of 1 Mm [4].

In order to investigate the mechanism of apoptosis in MCF7 lines we examined the activation of mitochondrial membrane potential and caspase-8. There are two main pathways by which apoptotic cell death can occur: The intrinsic (or mitochondrial) pathway and the extrinsic (death receptor) pathway [10]. Caspases are central regulators of the apoptotic process and are involved in both apoptosis pathways. CS produced no reduction in the red or green fluorescence generated by JC-1, suggesting a mitochondrial-independent pathway.

Since breast cancer is a malignancy with high metastatic potential, we examined the expression of cluster differentiation (CD) markers after CS treatment given that they have been implicated with migration, invasion and metastasis in several malignancies. Altered CD expression after chemotherapeutic agents administration has been associated with drug resistance and poor prognosis. Sun

et al. reported that CD44+/CD24- breast cancer cells isolated from MCF-7 cultures had substantial pro-angiogenic potential and activity which was abolished after treatment with bevacizumab [11]. The CD44+/CD24- cells have been propsed to be cancer stem cells (CSCs) [12] and are resistant to chemotherapy [13] and radiotherapy [14]. In the present study we found that CS reduced the expression of both CD24 and CD44 thus affecting CSC cells.

In addition, the present study showed that CS treatment of MCF7 cells reduced the CD58 and CD71 expression. Basal-like breast cancers (BLBC) are more common in young patients and are generally triple-negative tumors, thus they display aggressive clinical course, develop distant metastases and have unfavorable prognosis [15]. CD58 is among the basal-type associated genes [16], thus CS may have a role in the treatment of these difficult-to-treat subtypes of breast cancer. CD71 has been reported as a marker of poor prognosis in breast cancer and a candidate marker of a subgroup of ER+/luminal-like breast cancer that are

associated with poor outcome and resistance to tamoxifen [17]. CS significantly suppressed CD71 expression in the MCF7 cell line. Regarding, CD56 and CD15 expression no significant alteration was found after CS treatment.

In conclusion, breast cancer constitutes an heterogeneous malignancy, comprised of both differentiated and stem cells. A multifaceted approach combining several treatment strategies might be eventually required for better results. The present study indicated that exposure of breast cancer cells to CS significantly reduces cell viability, induces cell cycle arrest and apoptosis. Further studies are obviously needed to elucidate the complete mechanism of its apoptosic activity. Overall, CS seems to be a promising compound against breast cancer and there is a need our preliminary observations to be validated in breast cancer xenograft models.

Conflict of interests

The authors declare no conflict of interests.

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