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ORIGINAL ARTICLE

Antiproliferative and cytotoxic action of N-(p-coumaroyl) serotonin in lung cancer cells

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Summary

Purpose: *Lung cancer is among the leading causes of cancer-related cases and cancer-associated deaths. Tumor cells frequently acquire chemoresistance and, due to that, new therapies are always needed in the fight against cancer. Pharmaceutical plants continue to offer novel compounds as anticancer therapies.*

Methods: *We studied the action of N-p-coumaroyl-serotonin (CS), a natural compound from Centaurea seed and safflower on a lung adenocarcinoma cell line. Cytotoxic or antiproliferative effect was studied using the MTT assay. Cell cycle, caspase-8 activation, mitochondrial membrane potential (MMP) and expression of CD15/CD56/CD24/CD44/CD58/CD71 were studied by flow cytometry.*

Results: *CS exerted antiproliferative and cytotoxic activity, independent of mitochondrial membrane disruption. This compound caused S phase arrest and a decrease in the expression of CD24/CD44/CD58/CD71.*

Conclusion: *This is the first report on the in vitro action of CS against lung cancer, necessitating further studies towards its use as a potential anticancer agent.*

Key words: *anticancer agents, cytotoxic, lung cancer, N-(p-coumaroyl) serotonin*

Introduction

Cancer is among the leading causes of mortality, accounting for ~8.8 million deaths in 2015. Lung cancer, is among the most common types of cancer and also a major cause of cancer-associated mortality, accounting for 1.69 million deaths [1]. Almost half of new diagnosed patients are in advanced cancer stages, making them candidates for chemotherapy. Nevertheless, 2-year survival rates are less than 20%, because lung cancer cells frequently acquire multidrug chemoresistance [2].

The study of compounds from pharmaceutical plants is a major source of agents with anticancer activity [3]. N-(p-coumaroyl) serotonin (CS) is a serotonin conjugate, found in various plant species, including of *Centaurea* seed and safflower [4]. Studies on CS have shown that it has anti-inflammatory, antioxidative and fibroblast proliferation activity [4-7]. In two recent studies from our group, CS extracted from *Centaurea vlachorum* [8] exhibited antineoplastic activity against glioma cell lines [6]

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and cytotoxic action against a breast cancer cell line [9]. In the present study, we investigated the potential cytotoxic action of CS on the H1299 lung cancer cells.

Methods

Cell lines and treatment conditions

The human lung cancer cell line H1299 was obtained from ATCC (ATCC CRL-5803). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Grand Island, NY). The medium was supplemented with 10% fetal bovine serum (FBS), 100 μg/mL of streptomycin (Gibco BRL) and 100 units/mL of penicillin. Cells were grown at 37°C in a 5% CO_2 atmosphere. CS was dissolved in DMSO to acquire stock solutions. Increasing concentrations of CS were used to treat cell cultures.

Viability assays

Cell viability of H1299 cells, treated with various concentration of CS, was evaluated by trypan blue exclusion assay [10] and MTT assay [11], using untreated cells as control.

Flow cytometric analysis of DNA cell cycle

Flow cytometric analysis for propidium iodide (PI) was performed at 72 hrs. For DNA cell cycle analysis, first cells were trypsinized, centrifuged and washed with phosphate buffered saline (PBS). Thereafter, cells were incubated with PI-working solution (50 μg/mL PI and 20 mg/mL RNase A and 0.1% Triton X-100) at 37°C in the dark for 20 min. The PI fluorescence of 10,000 individual nuclei was calculated using a flow cytometer (FACScalibur, Becton Dickinson San Jose, California, USA). Using the mean peak fluorescence we determined the fractions of the cells in G0/G1, S, G2/M and sub-G1 phase with the CellQuest software (BD Biosciences, San Jose, CA, USA).

Flow cytometric analysis of caspase-8 activity

Activity of caspase-8 was determined with the Fluorescein Active Caspase-8 Staining Kit (Abnova, Taiwan) in treated and untreated cell lines, based on the manufacturer's protocol. Briefly, cells were trypsinized and 1 μl of FITC-IETD-FMK was added and incubated at 37°C, in an incubator with 5% CO_2 for 1 hr. Flow cytometry was used for quantification of fluorescent cells.

Flow cytometric analysis of mitochondrial membrane potential ($\Delta\Psi n$)

Mitochondrial membrane potential (MMP) was analysed using BD mitoscreen Mitochondrial Membrane Potential Detection kit, according to manufacturer's protocol. Cells were stained with JC-1 according to the protocol and analyzed with flow cytometry. Green and red fluorescence of cells was analysed to distinguish intact cells with high MMP to those undergoing apoptosis and significantly lower MMP.

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

Cells were treated with CS at concentrations of 100, 200, 300 and 400μM. As negative control used were nontreated cells. First, cells were dissociated by trypsinization and washed twice with PBS. Second, for blocking the Fc receptors, cells 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 CD71 (M-A712), FITC Mouse Anti-Human CD15 (W6D3) and PE Mouse Anti-Human CD71 (M-A712) antibodies (all from BD Pharmingen, San Diego, CA, USA) were added and in-

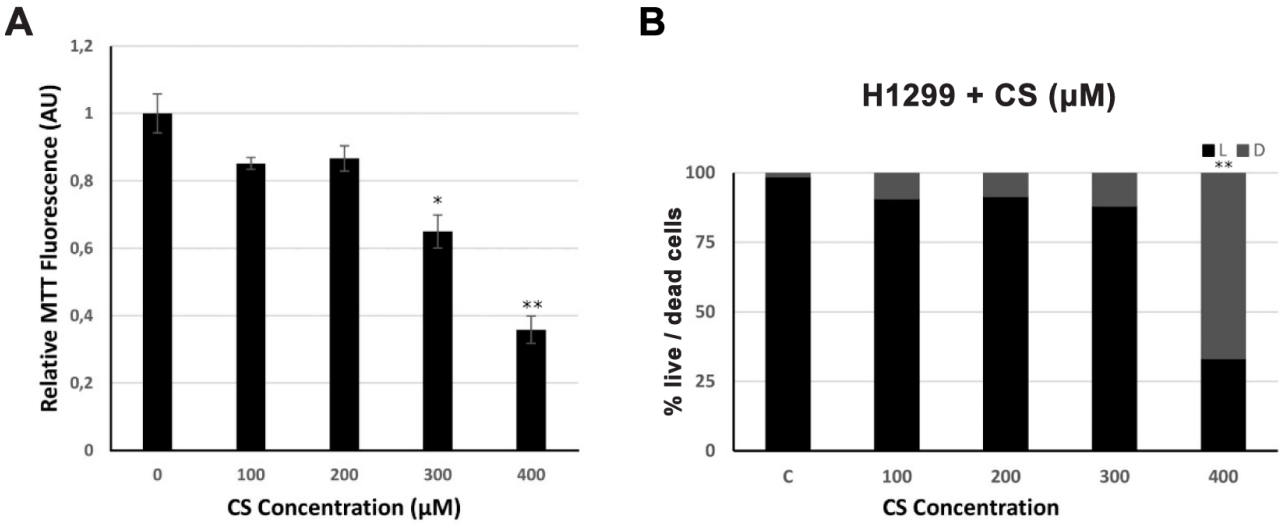

Figure 1. A: Results of the MTT assay after CS treatment on H1299. **B:** Viability of lung cancer cells following CS treatment. Cell viability was assessed by the trypan blue exclusion test in H1299 cells. 10^4 lung cancer cells were seeded in a 24-well plate. Twenty-four hrs after seeding, cells were treated with CS (100, 200, 300 and 400μM). Viability tests were performed 72 hrs post-treatment. Values are normalized to nontreated cells. (L=alive, D=dead). *p<0.05, **p<0.01.

cubated on ice in the dark for 20 min. Then, cells were washed twice with PBS and after resuspention in PBS they were analysed by a flow cytometer to achieve quantitatation of bound anti-CD24, anti-CD44, anti-CD15, anti-CD56, anti-CD58 and anti-CD71.

Statistics

Data are expressed as mean±SD. The significance of differences between experimental conditions was calculated using Student's t-test. Differences were considered significant at p values less than 0.05.

Results

IC_{50} calculation of CS effect on H1299 cells

In order to find whether CS had a cytotoxic or antiproliferative effect on H1299 cells, MTT assay was used. To that, H1299 cells were treated with CS (100-400 μM), following staining with MTT reagent. CS was found to induce cytotoxicity only at the higher concentrations used and 400 μM of CS resulted in reduction of MTT fluorescence up to 65%. IC_{50} values based on MTT assay were 346 μM during 72-h treatment (Figure 1A). To verify the cytotoxic effects of CS with an independent method, trypan blue exclusion test was used after CS treatment. We determined the cell survival rate, as the number of living cells at 72 hrs after exposure to escalating doses of CS compared with the number of live control cells. H1299 cell number decreased significantly with escalating doses of CS and the IC_{50} of cell cytotoxicity, based on trypan blue exclusion was at 343 μM, similar to that of MTT assay (Figure 1B). Cytostatic and cytotoxic effects have

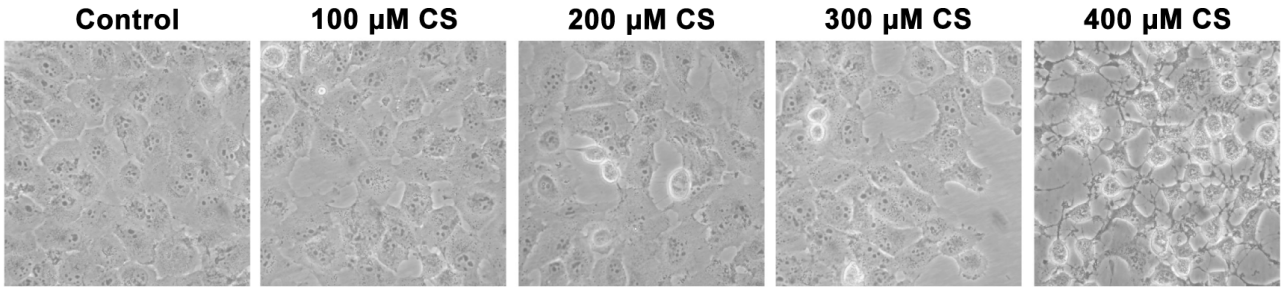

Figure 2. Phenotypic effects of escalating concentration of CS in H1299 cells (x20). CS treatment led to a dose-dependent induction of cytostatic (less confluent cells) and cytotoxic effects (induction of round cells).

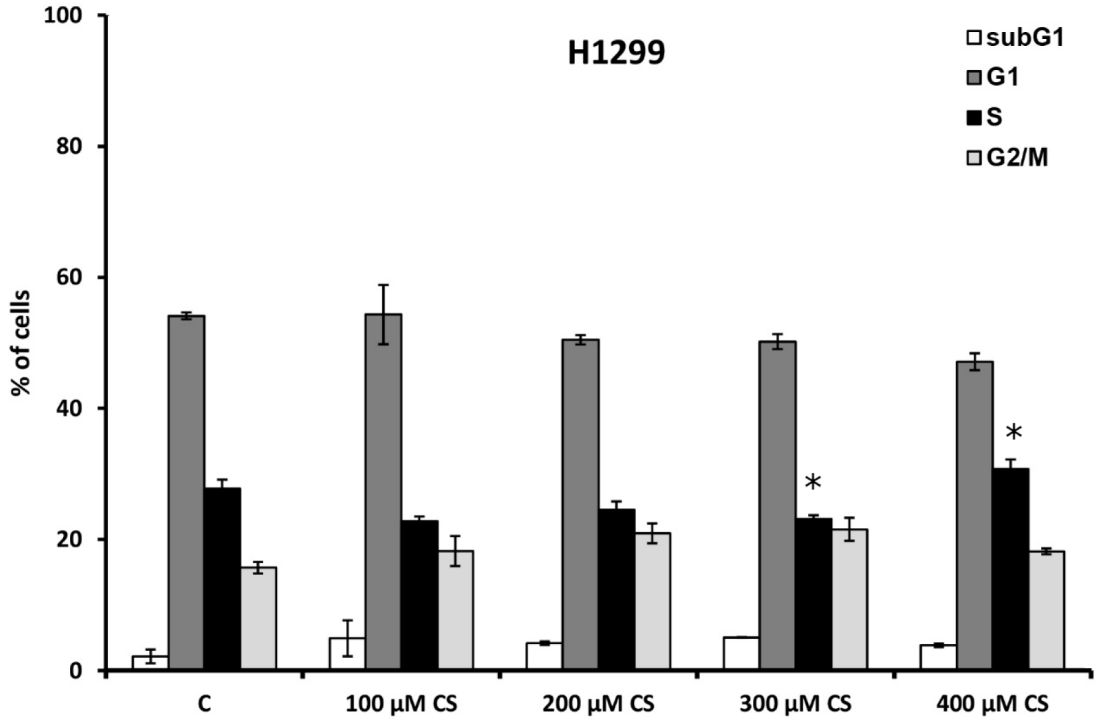

Figure 3. The effect of CS on cell cycle. H1299 cells ($2x10^5$) were seeded in 6-well plates and after 24 hrs were exposed to escalating concentrations of CS (100, 200, 300 and 400μM) for another 72 hrs. At 72 hrs, cells were stained by propidium iodide and the DNA content was evaluated by flow cytometry. CS produced S-phase cell cycle arrest dose-dependently. *p<0.05.

also been confirmed with microscopic observation, revealing that CS resulted in a dose-dependent cytostatic action and also cytotoxic effects (as round cells), more prominent at 300 and 400 μM (Figure 2).

CS induced cell cycle arrest

To assess the effect of CS on cell cycle progression of H1299 cells, we performed cell cycle analysis by flow cytometry, following CS treatment (100, 200, 300 and 4200μM) for 72 hrs and we found that, while low concentrations did not result in a significant change on cell cycle distribution, 300 μM and 400 μM of CS resulted in a S-phase cell cycle arrest (Figure 3).

CS increased the activation of caspase-8

Next, we investigated the effect of CS in caspase-8 activation, after treatment with 200 and 400μM of CS. While at 200 μM there was no significant caspase 8 induction, at 400 μM CS produced significant higher activity of caspase-8 compared to control, indicating induction of apoptosis (Figure 4).

CS resulted in altered expression of various CD markers

We then analysed the expression of CD15, CD56, CD24, CD44, CD58 and CD71 by flow cytometry. While CD15 and CD56 were not expressed in H1299 cells, alteration to CD marker expression has been observed, with 400 μM of CS resulting in

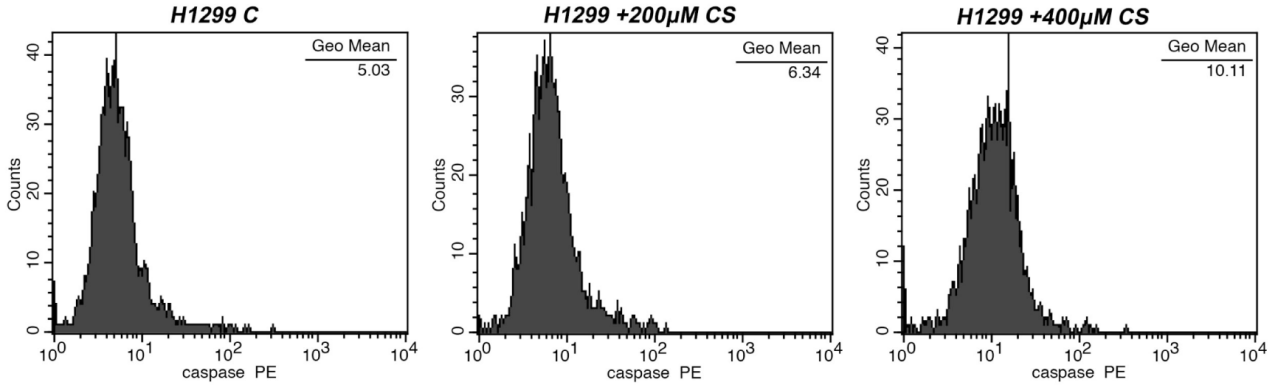

Figure 4. Representative histograms showing the activation of caspase-8. H1299 cells (10^5) were seeded in 24-well plate and after 24 hrs were exposed to CS (200 and 400μM) for another 24 hrs. A significant increase in activated caspase-8 was observed after treatment with CS.

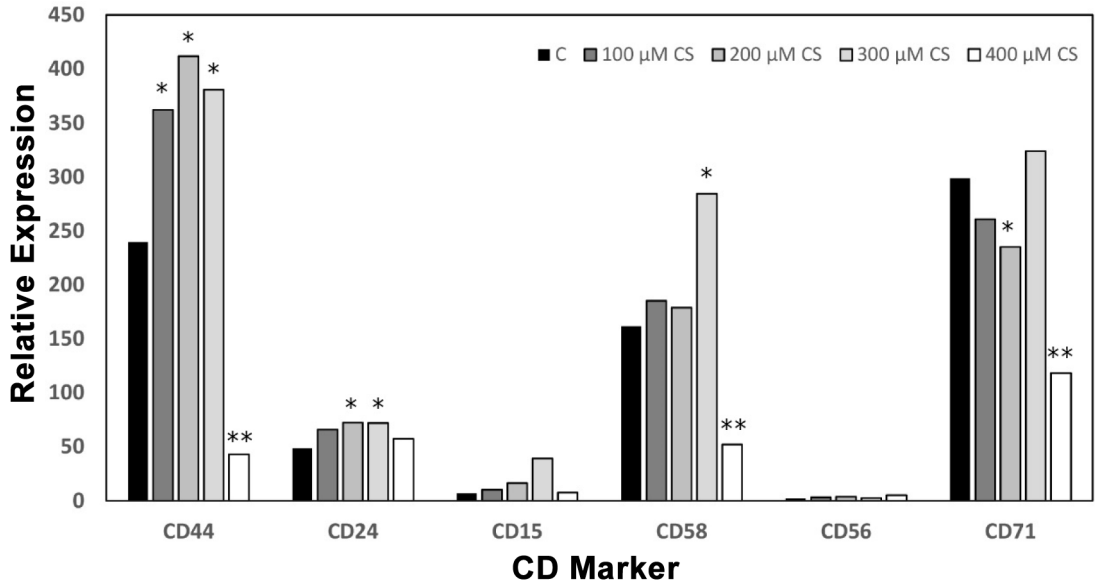

Figure 5. Flow cytometry analysis for the expression of CD24/CD44/CD56/CD58/CD15/CD71 in H1299 cells. H1299 cells (10^5) were seeded in 24-well plates and after 24 hrs were exposed to escalating concentrations of CS (100, 200, 300 and 400μM) for another 72 hrs. There was a dose-dependent decrease in CD24, CD44, CD58 and CD71 expression. No significant change was noted for the CD56 and CD15 expression after CS treatment. *p<0.05, **p<0.01.

a significant decrease in the number of CD24/CD44/CD58/CD71 molecules per cell (Figure 5).

Discussion

The present study showed that CS may have a potent activity in lung cancer cell lines *in vitro*. CS induced suppression of lung cancer cell growth, S-phase cell cycle arrest and apoptosis in H1299 cell line, which was also associated with activation of caspase-8 and mitochondrial-independent action. The higher concentration of CS also produced decreased CD44/CD24/CD58 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 via apoptosis induction and cell cycle arrest at G2/M and S-phase [6]. 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 [6].

In order to investigate the mechanism of apoptosis in H1299 cell line we examined the two main pathways by which apoptotic cell death can occur: The intrinsic (or mitochondrial) pathway and the extrinsic (death receptor) pathway [7]. CS produced no reduction in the red fluorescence or green fluorescence generated by JC-1, suggesting a mitochondrial-independed pathway, while activation of caspase-8 indicated an extrinsic pathway of cell death.

We examined the expression of cluster of differentiation (CD) markers given that they have been implicated with migration, invasion and metastasis in several malignancies. Lung cancer constitutes a heterogeneous tumor, comprised of both differentiated and stem cells. The expression of surface markers can be used to characterize cell phenotype and also, in some cases, to predict their response to chemotherapy. Additionally, altered CD expression after administration of chemotherapeutic agents has been associated with drug resistance and poor prognosis. As an example, CD44 and CD24 are well-established cancer stem cells markers, first identified in breast cancer but also expressed in other types of cancer [12]. In our study, the expression of CD24 remained unaffected by CS, while 400 μM of CS, a concentration near the IC_{50} (346 μM, based on MTT assay, Figure 1), resulted in a significant reduction of CD44. Since CD44 is a surface molecule that can lead to cancer progression, reduction of CD44 may be an additional anticancer action of CS. CD58 is a novel marker associated with self-renewal of colorectal cancer-initiating cells [13]. In our study, we found that CD58 was also expressed in H1299 cells and also that CS resulted in CD58 reduction. CD71, also known as transferrin receptor, is a poor prognostic marker on breast cancer [14] and also a marker expressed in glioma cancer stem cells [15]. CD71 is also expressed in H1299 cells [16] and we have found that CS at 400 μM resulted in significant reduction of CD71. The reduction of these markers may be involved in the cytotoxic effects of CS on H1299 cells, but further analyses are needed to investigate the underlying mechanism.

Collectively, the present study indicated that exposure of lung cancer cells to CS reduced cell viability and induced cell cycle arrest and apoptosis. Additionally, our findings indicate a mitochondrial-independent pathway of apoptosis and reduction of cell surface markers CD24 and CD71. Further studies are obviously needed to elucidate the complete mechanism of its apoptotic activity. In any case, our results support a cytotoxic action of CS against lung cancer cells and necessitate further validation in lung cancer xenograft models.

Conflict of interests

The authors declare no conflict of interests.

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