A Study on the Combined Effects of Diallyl Sulfide, Cisplatin, and Celecoxib on Cervical Carcinoma HeLa Cells

Danay Rodriguez, Nataly Abrams, Hollie Canacari, Talal El-Hefnawy, M.D., Ph.D., Diana Schultz, Ph.D.
Florida Gulf Coast University, College of Arts & Sciences, Fort Myers, FL 33965

ABSTRACT
The American Cancer Society predicts that, in the U.S. alone, one third of the approximately 13,000 women diagnosed with cervical cancer in 2016 will not survive. Chemotherapeutic drugs, such as cisplatin, are known to induce cross-linkage of the DNA ultimately inhibiting cell replication. On the other hand, neoadjuvant therapy also exhibited efficacy in treating malignancies either alone or in combination with chemotherapy. Compounds, such as celecoxib, sensitize cancer cells to apoptosis through the cyclooxygenase-2 pathway. Although effective, drugs such as cisplatin combined with celecoxib are toxic to all cells, including healthy cells, provoking severe side effects, leading scientists to seek alternatives including natural food ingredients in the battle against cancer. A natural extract from garlic, diallyl sulfide (DAS), also proves to induce apoptosis in several cancer lines. Nevertheless, the effect of celecoxib and DAS in combination, as a replacement therapy to cisplatin, has never been studied in cervical cancer cell lines. This study focuses on the combined effects of celecoxib, DAS, and cisplatin on cervical carcinoma HeLa cells at LD25 and LD50 concentrations following 24 and 48 hour treatment. Our results of DNA gel electrophoresis analysis showed no indication of apoptosis at 24 hours with cisplatin alone, whereas a clear DNA ladder (indicative of apoptosis) was observed 48 hours after treatment. Similar results were also observed when studying the synergistic effects of cisplatin and celecoxib at LD25. When the concentration for both chemicals was raised to their LD50, irrefutable evidence of apoptosis was observed as early as 24 hours after treatment.

Key words: cervical cancer treatment, celecoxib, cisplatin, diallyl sulfide, DAS, apoptosis

INTRODUCTION
The American Cancer Society predicts that, in the U.S. alone, one third of the estimated 13,000 women diagnosed with cervical cancer in 2016 will succumb to the disease. Although these numbers are alarming they are significantly lower than earlier years due to the development of two preventive vaccines, Gardasil® and Cervarix®, which have managed to reduce the risk of contracting certain types of the human papilloma virus (HPV) associated with cervical cancer. Nevertheless, those women who are not protected and contract cervical cancer are left with harsh treatment options including surgery, radiation, and chemotherapy, depending on the type and stage of the disease.

Prostaglandins (PG) are important lipids responsible for maintaining homeostatic functions in the human body. The enzyme cyclooxygenase-1, (COX-1), termed “the housekeeping enzyme,” is expressed in all human tissues and one of its functions is the production of prostaglandins (PG). In contrast, COX-2 is not present in all tissues and its function is strictly regulated by the cell so that under normal conditions it is not expressed. When a tissue is inflamed, the COX-2 pathway is activated. Here the enzyme catalyzes the conversion of arachidonic acid into prostaglandins E2 (PGE2). It has been identified as the principal prostanoid that promotes cell growth and survival in some tumors. The production of PGE2 appears to be a direct consequence of the upregulation of COX-2 in inflamed tissue, such as cancer, but more specifically in cervical cancer. With this observation, scientists suggest that inhibition of this enzyme will diminish tumor progression and growth.

The chemotherapeutic drug cisplatin is an alkylating agent that can bind to DNA and interfere with the cell’s repair mechanism, ultimately leading to cell death. Treatment with this chemotherapeutic drug, however, increases the cytotoxicity for all cells, not only cancer cells, and provokes severe side effects to the patient, such as nausea, vomiting, and diarrhea. Therefore, it is essential to explore alternative methods of treatment such as combination therapy with other less aggressive drugs such as celecoxib and DAS. Celecoxib is a well-studied drug and a known COX-2 inhibitor. In observation, the drug sensitizes the apoptotic pathway taken by cisplatin to interfere with the cell’s repair machinery.

The natural garlic plant extract diallyl sulfide (DAS), a compound with similar anti-tumor activity as cisplatin, also interferes with COX-2 expression in tissues, thus reducing inflammation. DAS is documented as a potential chemotherapeutic agent as it proves to induce apoptosis in human colon, prostate, breast, bladder, and leukemia cancer lines, among others. Nevertheless, the interactions between celecoxib and DAS have never been studied in combination in cervical cancer cell lines.

This study focuses on the individual interactions of both DAS and cisplatin against the selective COX-2 inhibitor, celecoxib, with regards to triggering apoptosis on cervical cancer HeLa cells.

METHODS
Reagents and Chemicals
HeLa cells, CCL-2, were obtained from ATCC (American Type Culture Collection, Manassas, VA.). Celecoxib 99% (TSZ Chem), Cisplatin powder (Tocris Bioscience), DAS 97% (Acros Organics), DMSO 99.9% (SIGMA),
Corn Oil (Fischer Scientific Education), Agarose (Fisher Bioreagents), DMEM, 1X (Corning), Phosphate-buffered saline (PBS, Corning) and Trypsin 0.05% 0.53mM EDTA (Corning) were purchased from Fisher Scientific (Waltham, WA). The Wizard SW genomic DNA Purification kit, and the DNA marker (1kb) were obtained from Promega, and Gel-Red™ from Phoenix Research Products (Candler, NC). LD50 start point concentrations for Cisplatin (11µM), Celecoxib (10µM), and DAS (10µM) were according to the literature.  

**Cell Viability**

HeLa cells were seeded onto six-well culture plates, allowed to attach for 24 hours, and then treated with the corresponding drugs (celecoxib, DAS, or cisplatin, alone or in combination). After 24 or 48 hours, the supernatant was removed and the cells were washed with 1mL of ice cold PBS. 1mL of trypsin was then added per well to detach cells from the plates. Once completely detached, 500µL of DMEM was added to the washed cells to counteract any effects trypsin may have on the cells. After detachment, 50µL of cell suspension was collected and combined with 50µL of trypan blue dye (0.4% w/v, SIGMA), to differentially stain dead cells. 10µL of this cell suspension was loaded onto a hemocytometer and live cells counted under a contrast phase microscope.

**Lethal dose (LD) determination**

HeLa cells were seeded onto six-well tissue culture plates, allowed to attach for 24 hours and then exposed to various concentrations of cisplatin, celecoxib and DAS alone or in combination for an additional 24 hours. After treatment, cell viability was assessed by the method described above. After three independent experiments, the average cell count was calculated and graphed, and individual lethal dose 25 (LD25) and LD50 were calculated for each chemical. The regression analysis performed on Excel provided a trend line between cell viability and drug concentration. The software generated equation was used to determine the concentrations that kill 25% (LD25) and 50% (LD50) of the cells. Combination of the chemicals at the corresponding individual calculated LD25 and LD50 concentrations were used to study the interactions of two drugs at a time.

**DNA Laddering**

HeLa cells were cultured on six-well plates and allowed to attach for 24 hours, they were then treated with LD25

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**Figure 1:** Effect of DAS (A), cisplatin (B) and celecoxib (C) on cell viability. Linear regression analysis (shown) was used to calculate each of the drugs LD25 and LD50 concentrations. DAS did not follow a linear pattern throughout the experiments, therefore, it was not studied any further.
and LD50 concentrations of cisplatin, celecoxib and DAS, alone and in combination (celecoxib and cisplatin, celecoxib and DAS). After exposure to treatment for 24 and 48 hours, cells were washed with ice cold PBS and re-suspended in Wizard® SV Lysis Buffer (Promega, Madison, WI). Cell DNA was then extracted using the Wizard SV genomic DNA purification system, according to instructions from the manufacturer and as previously described. Electrophoresis was performed on 1% agarose gel in combination with the nucleic acid stain Gel-RedTM. This aided the visualization of DNA (1μg/ lane) under UV light. The gels were then photographed.

**Prostaglandin assessment**

After 24 hours of treatment, cell media was collected. 2mL of cell media was combined with 2mL ethyl acetate and loaded onto a vortex to allow fusion of the two phases. The resulting mixture was centrifuged for 1 minute at 1,000 rpm and after separation of phases the organic layer was collected and transferred to a new test tube, vacuum-dried, and re-suspended in methanol. Afterward, the sample was analyzed by liquid chromatography mass spectrometry using TSQ triple quadrupole mass spectrometer coupled with HPLC as front end. Assessments of prostaglandins were done using electrospray ionization using negative ion mode.

**RESULTS**

**LD25 and LD50 concentrations at 24 hours for cisplatin, celecoxib and diallyl sulfide**

Results showed that, at 24 hours, the LD25 concentrations for cisplatin and celecoxib were 16.9μM and 10.34μM and that their LD50 concentrations were 38.7μM and 22.94μM, respectively, (Fig. 1B and Fig. 1C). DAS, on the other hand, did not follow a linear trend (Fig. 1A), therefore, it was decided to not proceed with this chemical.

**Synergistic effects of Cisplatin and Celecoxib on cell viability**

Using both chemicals’ LD25 concentrations for 24 and 48 hours, the synergistic effects were analyzed. At 24 hours, combination therapy reduced cell viability by 5.2% when compared to the control; at 48 hours, results show 48.3% reduction of cell viability. The synergistic effects were further investigated using both chemicals at LD50 concentrations for 24 and 48 hours. Results showed that, at 24 hours, cell viability was reduced by 48.1%, and at 48 hours it was reduced by 81.8% (Fig. 2).

**DNA ladder formation, a hallmark for apoptosis**

DNA ladder formation was assessed through agarose gel electrophoresis of genomic DNA purified after treatment with LD25 and LD50 concentrations of cisplatin, celecoxib, and DAS alone and in combination (celecoxib and cisplatin, celecoxib and DAS). Gel electrophoresis of DNA extracted from cells after 24 hours of treatment with cisplatin alone, at LD25 concentration, did not show evidence of apoptosis (Fig. 3B), however, DNA ladder- ing was evident at 48 hours (Fig. 3D). Combination of cisplatin and celecoxib LD25 concentrations showed no
apoptosis at 24 hours (Fig. 3C). However, at 48 hours, DNA laddering was observed (Fig. 3E). Finally, when the concentration of both chemicals was raised to their LD50, evidence of apoptosis (DNA laddering) was observed as early as 24 hours after combination treatment (Fig. 3F-I).

**DISCUSSION**

Previous research on several cancer cell lines have attempted to investigate the pathway taken by celecoxib and cisplatin, suggesting celecoxib acts as a chemosensitizer to cancer cells. A publication looking directly at the interactions between cisplatin and celecoxib in different cancer cell lines, including cervical cancer, agreed with our findings and showed modification of the cell cycle by celecoxib. Based on previous research findings, such as the one mentioned above, a hypothesis was formulated, which consisted of replacing the chemotherapeutic drug, cisplatin, with the natural plant extract diallyl sulfide and the nonsteroidal anti-inflammatory drug celecoxib. Results of our study indicate that cisplatin alone and in combination with celecoxib induced apoptotic effects on cervical carcinoma HeLa cells. Our results indicate that a reduction of cell viability is correlated with increased drug concentration; yet, this was not the case when observing DNA ladder formation, which was evident after 48 hours of treatments at LD25 and LD50 concentrations. In addition to ladder formation, a hallmark for apoptosis, PGE2 production was assessed using mass spectrometry as a method of determining COX-2 expression in the cells. However, the results were inconclusive and should be carefully repeated. Our results on DAS treatments, alone, were also inconclusive because cell viability did not follow a linear regression. This pattern should be further analyzed in future studies that will allow understanding of how DAS is interfering with the cell cycle at various concentrations and time intervals. Previous research on a molecule similar to DAS, diallyl disulfide (DDAS), shows similar behavior on lung carcinoma. After a clear understanding of DAS behavior on HeLa cells, the experiment must proceed and focus on interactions between DAS and celecoxib. These results should provide some insight into a potential drug combination therapy, the interactions of which have never been recorded on a cervical cancer cell line.

**REFERENCES**