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Cellular and Molecular Biology Effect of Carboplatin and Doxorubicin on Human Endometrial Epithelial Hec-1-B Cell Cultures

KEYWORDS: Carboplatin, Doxorubicin, HEC-1-B cell line.

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ABSTRACT *Chemotherapy is the main approach in dealing with advanced and recurrent endometrial cancer. This study evaluated the antitumor effects such as carboplatin and doxorubicin on the human endometrial epithelial HEC-1-B cell line. HEC-1-B cells were allowed to grow in DMEM/F12 media with 10% fetal bovine serum and treated with the compounds mentioned above. The target of this research was to identify the exact concentration of two components that HEC-1-B cells were affected in. In the apoptosis assay, it was found that the minimum concentration to induce apoptosis was 2,220 nM. Moreover, a soft agar assay was used to perform which concentration of carboplatin and doxorubicin had the more colonies of cells.*

Introduction

With improvements in tissue culture techniques, numerous human cancer cell lines have been established and developed which can enable us to perform further studies for such cell lines, and make it handy to learn more about cancer diseases. Consequently, a better chance we would conceive to discover more medications and treatments to cure these cancers.

In this study, human endometrial epithelial HEC-1-B cell line has been used in many experiments such as "Effect of compounds on cell viability" and "Effect of compounds on apoptosis".

Endometrial cancer is a significant cancer type relates to the uterus where abnormal growth of cells takes place in causing cancer. The most common cause of endometrial cancer is having too much of the hormone estrogen compared to the hormone progesterone in the body (WebMD, 2012). Endometrial cancer is characterized by a variety of genetic alterations, but the most common alteration is in the PTEN gene which located at chromosome 10q23, encodes a protein and lipid phosphatase which behaves as a tumor suppressor gene (Tsuyoshi Okuda, Akihiko Sekizawa, 2010). Endometrial cancer is a popular cancer disease among women, with an estimated 47,130 new cases expected to happen in the US in 2012. In fact, Endometrial cancer has being observed through a number of studies and researches pointing out this disease targets women with an average age of 60 years. According to the National Cancer Institute, it is estimated that approximately 8,010 women in the United States will die of endometrial cancer in 2012 (National Cancer Institute). Most cases of endometrial cancer are usually discovered in its early stages with a good diagnosis for the overall 5-year survival rate in 80% of patients, yet there is about 10% to 20% of cases are discovered in the early stages with lower 5-year survival rates. Multimodality therapy is considered the main cure for advanced endometrial cancer cases, with radiation and chemotherapeutic regimens being frequently combined. Nowadays, combination chemotherapy such as doxorubicin and carboplatin are well accepted for treating endometrial cancer (Cheng-Jeng Tai, Chien-Kai Wang).

Carboplatin belongs to the set of medications that are known as alkylating agents, and it is used vastly as a cure for the cancer of the ovaries specially if it is modified with other components such as docetaxel (Bruchim, Ilan MD; Weeg, Natalie MBBS,2016). It might be also introduced as a good treatment option for some other kinds of cancer. Generally, Carboplatin is meant to deal with the growth of cancer cells which basically are harmed and damaged (MAYO CLINIC).

Carboplatin is formed by replacing the chloride leaving groups of cisplatin with 1, 1-cyclobutanedicarboxylato ligand, which increases the stability of the leaving groups. The major antineoplastic mechanism of action for carboplatin is the production of cross links within and between strands of deoxyribonucleic acid (DNA). Normal DNA synthesis is inhibited by this disruption of cellular DNA conformation. Carboplatin *is more soluble and chemically stable and binds more slowly to plasma proteins (*CHEMICALLAND21*).*

Doxorubicin is usually related to a common list of drugs that are known as antineoplastics. It is used as a treatment for some types of the blood malignancies, such as lymph system, bladder, breast, stomach, lung, ovaries, thyroid, nerves, kidneys, bones. And also it is used for smooth cells, such as muscle tissue and muscle (MAYO CLINIC).

Doxorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. This prevents the chain of the enzyme topoisomerase II, which stops DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.

The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA, while the six-membered daunosamine sugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures (US Biological).

Carboplatin and Doxorubicin have been chosen in this study since many studies show these components have a significant effect on HEC-1-B cells (William B. Grant, 2010). Furthermore, more researches have been approached showing that those components are the most components that could be used as treatments for endometrial cancer (Paul J. Hoskins, Kenneth D. Swenerton, 2001), (M. S.

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Aapro, F. H. van Wijk, 2003). For Carboplatin, in addition, it has been proven that the Carboplatin has the highest rate for treating endometrial, and it has become a standard therapy in these days (Paul J. Hoskins, Kenneth D. Swenerton, 2001).

Carboplatin and Doxorubicin's ability to induce apoptosis have been also used in this study. Apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. When apoptosis does not work correctly, cells that should be eliminated may persist and become undying.

The purpose of this research is to know the exact effect of Carboplatin and Doxorubicin on HEC-1-B cells and to conclude if they have a perfect impact on HEC-1-B or not. Moreover, this study has been approached to determine the ED50 for the two components in which they have been used separately. In addition, to know if Carboplatin and Doxorubicin are capable to make apoptosis or not, and to know the great concentration of the two drugs that impacts on HEC-1-B cells.

Materials and Methods

Preparation of Compounds

MTT, Cytotoxicity Assay. Assay was done in a 48-well plate. Sterile micro-tube strips were used to make the dilutions of the compounds. Dilution of the compounds were initially done with DMSO and then with the media. The group was provided with 100 µl of the 1000x stock in DMSO for each compound in the first tube of a sterile strip.

A 1:3 dilution series in DMSO of doxorubicin (30,000 nM) and carboplatin (60,000 nM) were prepared. Concentrations for doxorubicin ranged from 2 nM to 30,000 nM. Concentrations for carboplatin ranged from 3 nM to 60,000 nM. The cells were plated in growth media and allowed to attach prior to treating with compounds. Each well contains 0.5 ml. The total number of cells/well was 20,000.

To determine the cytotoxicity of the compounds, the cells were stained with MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5, diphenyltetrazolium bromide) for 3 hours. MTT was reduced by mitochondrial activity producing a precipitate that is dark purple. 100 µl of 5 mg/ml MTT stock solution was added to the 1 ml of media already in each well, except the three "Blank" wells. The plate was placed in the incubator (37°C) for 3 hours.

At the end of the 3-hour incubation, the media was removed from each well. 500 µl DMSO and 25 µl Sorensen's glycine buffer was added to each well. The stained plate was allowed to develop for 10 minutes at room temperature with gentle shaking. The absorbance due to the dye was read on a microplate reader at 570 nm. Excel was used to analyze the data and the results were graphed to determine the ED50. P- values were calculated for each value using t-test Two – sample Assuming Equal Variances.

Apoptosis Assay. For this assay 40,000 cells/ml were used. One 8-well microscope slide was set up; each well held 0.5 ml with concentrations of carboplatin ranging from 80 nM to 60,000 nM. The slide was labeled and placed in the incubator. The slides were treated with the compounds for one day. The medium on the cells was removed and replaced with growth medium containing Hoechst and Propidium Iodide then placed back in the incubator for 1 hour. After the incubation with the dyes, the slide was washed with non-sterile PBS and prepared for fluorescence microscopy.

The slides were then mounted with a coverslip and covered with foil to prevent fading of the signal. Photographs were taken of three random fields per well using a 20x objective. In order to see both the Hoechst and Propidium Iodide staining, the field was initially photographed with the "DAPI" filter in place, which shows the Hoechst stained nuclei as blue, then without moving the stage, photographed with the "Red" filter in place, which shows the Propidium Iodide stained nuclei as red. Forty eight photographs were taken. A combination of the DAPI image with the Red image was used to produce 24 images with blue nuclei in healthy cells and purple apoptotic nuclei.

Counts and Calculations. For each of the groups there were 24 merged images. The total number of nuclei in each field and the number of purple nuclei in each field were counted. For each merged image the % Apoptotic cells and the ED50 was calculated using Excel.

Soft Agar Assay

Plate Preparation. This assay was performed to determine the number of colonies HEC-1-B cell lines formed in the presence of Carboplatin. A 6-well plate was prepared; the plate contained 1.5 ml of growth media with 0.5% agar. The plates were stored at 4°C for 2 hours. Two wells were prepared with just DMSO for use as controls. The cell density in the cell suspension used was 40,000 cells/ml.

Growth Media with Soft Agar Preparation. A tube with 3.5 ml of 2x Growth Media from the 42°C water bath was obtained and 7 µl of Carboplatin (1:3 dilution; 20,000 nM and 1:81 dilution; 740 nM) or DMSO was added to the 2x Growth Media. The 2x Growth Media tube was returned to the 42°C water bath, while one plate with bottom agar was obtained from the incubator and labeled. Three ml of 2x Growth Media were added to the 3 ml of 1.0% agar. Then 0.1 ml of cell suspension was added to the agar mixture, this was mixed gently and 1.5 ml were added to each well to two corresponding wells of the 6-well plates. These steps were repeated for the second and third set of wells. The plate was returned to the incubator.

Detection of Colonies. The number of colonies in the HEC-1-B cell lines formed in the presence or absence of your compounds was determined as follows:

The staining was done by adding 0.5 ml of 0.005% Crystal Violet to each well. Incubation at room temperature for 1 hour was followed. Then grids were drawn on the lid of the multi-well plate with a fine point marker, the grids were about 0.5 cm. The plates were placed on a dissecting microscope with the lid under the plate. At the highest magnification possible, colonies were counted as any balls of cells consisting of more than 8 cells. Then the percentage of cells that formed colonies was reported.

Results

Cytotoxicity Assay

Values on Figure 1 show an ED50 value for carboplatin at a concentration equal to 6,000 nM. Low concentrations of carboplatin show no inhibitory effect. The MTT assay was used to measure percent viability as compared to control HEC-1-B cells (Figure 1). The cells were not inhibited by 3 to 2,220 nM Carboplatin. Inhibition of cells in concentra-

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tions ranging from 6,000nM to 20,000 nM of Carboplatin was observed (Figure 1). An insignificant increase in the dose curve after 20,000 nM is also noted (Figure 1). P-values obtained for all concentrations of Carboplatin were below 0.05 (Table 1).

Values on Figure 2 show an ED50 value for Doxorubicin at a concentration equal to 4,000 nM. Low concentrations of Doxorubicin show no inhibitory effect. The MTT assay was used to measured percent viability as compared to control HEC-1-B cells (Figure 2). The cells were not inhibited by 2 to 370 nM Doxorubicin. Significant inhibition by 1111 nM and higher concentrations of Doxorubicin was observed (Figure 2). P-values of concentrations 30,000, 10,000, 3333, 1111 and 2 nM were lower than 0.05. The remaining concentrations of doxorubicin including 370, 123, 41, 14 and 5 nM had p-values ranging from 0.1 – 0.5 (Table 2).

Apoptosis Assay

Results from images processed by ImageJ indicate percent of apoptosis induced by Carboplatin where, Blue and purple cells represent live cells and dead cells respectively (Figure 3). Apoptosis assay results show that a concentration equal to 2,220 nM of Carboplatin induces 100% apoptosis in HEC-1-B cells (Figure 3 and 4). In the absence of Carboplatin, a high apoptotic % was observed (Figure 4). Then a rise in apoptotic % followed by gradual decrease was noted as concentrations were increased at a constant rate (Figure 4).

Soft Agar Assay

Results from proliferative assay in soft agar were obtained with two different concentrations of Carboplatin (740 and 20,000 nM) compared with control (Figure 5). The lowest concentration of Carboplatin showed the highest number of colonies and the control had growth considerably greater than the highest concentration of Doxorubicin (Figure 5). Growth of cells was substantially inhibited at a concentration of 20, 000 nM showing low colony counts (Figure 5).

Significant results for proliferative assay in soft agar were obtained with two different concentrations of Doxorubicin (370 and 10,000 nM) compared with control (Figure 6). Control showed the highest number of colonies and the lowest concentration had growth considerably greater than the highest concentration of Doxorubicin (Figure 6). Growth of cells was substantially inhibited at a concentration of 10, 000 nM showing low colony counts (Figure 6).

Discussion

The various concentrations of Carboplatin and Doxorubicin were used in the experiment "Effect of compounds on cell viability" have an effect on HEC-1-B cells. For both components, we used 60nM, 20nM, 6.67nM, 2.22nM, 0.73nM, 0.24nM, 0.08nM, and 0.02nM concentrations with dilution 1:3. According to MTT assay figure 1, and figure 2, the highest concentration of both drugs (carpoplatin and doxorubicin) have an ordinary impact on HEC-1-B cells; however, the lowest concentration of both drugs have no effect on the cells. The major effect on the cells were at the range from 6,000 nM to 20,000 nM for carpoplatin while it was specific at 1111 nM for doxorubicin. The ED50 value for Carboplatin took place at the concentration 6,000 Nm, and the P-values gained for all concentrations of Carboplatin were 0.05. There is a study shows that the Carboplatin with the highest concentration is considered a great drug for endometrial cancer having an essential impact on HEC-1-B cells (William B. Grant, 2010). Furthermore, another study demonstrates that doxorubicin can affect on HEC-1-B cells at some various concentrations (Maki Ikeda, Akira Kurose, 2010). All studies mentioned above confirm that HEC-1-B cells could be influenced only with the highest concentration of Carpoplatin and Doxorubicin. According to a study entitled as "Antitumor Activity of Ridaforolimus and Potential Cell-Cycle Determinants of Sensitivity in Sarcoma and Endometrial Cancer Models" Crboplatin and Doxorubicin are one of the components that can reduce the rate of proliferation of endometrial cancer cell lines (Rachel M. Squillace, David Miller, 2011). As a result, Doxorubicin has a greater effect on HEC-1-B cells than Carboplatin contrary to what we expected at the beginning.

The effect of compounds on apoptosis presents that the only 2,220 nM concentration Carboplatin persuaded apoptosis in HEC-1-B cells.

A soft agar assay for Caboplatin which have been used in this research illustrated that the highest percentage of cells that produced colonies was at the lowest concentration (740 nM) of Carboplatin; however, the highest concentration of Carboplatin which was 20,000 nM had the least number of colonies. In addition, the control showed the highest number of colonies more that and the 20,000 nM Carboplatin. On the other hand, the soft agar assay for Doxorubicin showed that the highest percentage of colonies was in control compared to colonies which were in 370 and 10,000 nM concentrations of the drug. Therefore, that is an additional fact confirms that both components could play significant roles at the highest concentrations, and both are considered great drugs for treating endometrial cancer.

Mixing anything with the drug can contaminate the drug and change the results dramatically, so it is measured an error as well.

This kind of study provides that the only highest concentration of Carboplatin and Doxorubicin can inhibit HEC-1-B cells growth. On the other hand, this study clearly shows the promise of the lowest concentration of these components may treat the endometrial cancer in the future.

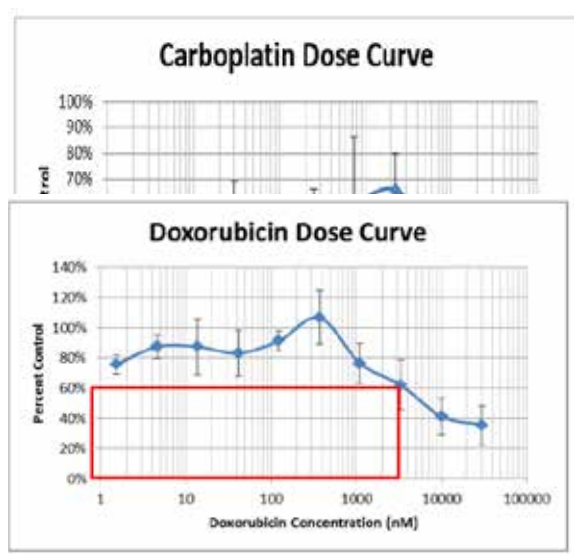

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Figure 2. Dose curve of HEC-1-B cells treated with Doxorubicin after 48 hours.

Carboplatin Conc. (nM)	60,000	20,000	6,670	2,220	740
P-values	0.0217	0.0079	0.0129	0.0457	0.0435
Carboplatin Conc. (nM)	250	80	30	10	3
P-values	0.0121	0.0039	0.0158	0.0039	0.0030

Table 1: Significant P- values for Carboplatin.

Doxorubicin Conc. (nM)	30, 000	10, 000	3333	1111	370
P-values	0.00033	0.00048	0.01223	0.04021	0.57708
Doxorubicin Conc. (nM)	123	41	14	5	2
P-values	0.20218	0.14435	0.31996	0.10275	0.00692

Table 2: Significant P-values for Doxorubicin.

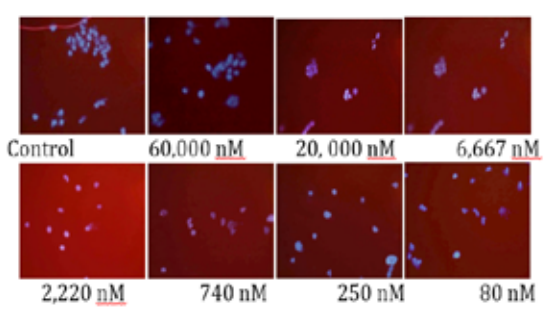

Figure 3. Apoptosis assay Images.

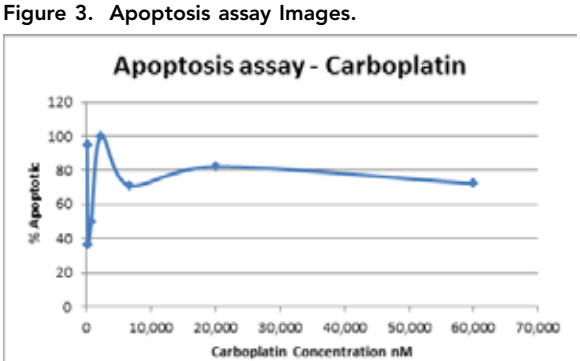

Figure 4. Apoptosis assay of Carboplatin treated HEC-1-B cells for two days –HEC-1-B cells incubated with Hoechst and Propidium Iodide dyes and photographed using the DAPI filter and the red filter. The pictures were processed with ImageJ (Figure 3).

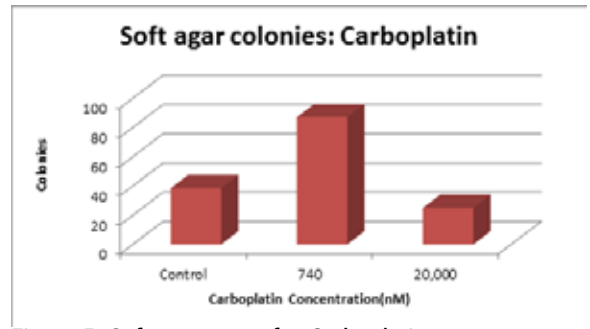

Figure 5. Soft agar assay for Carboplatin.

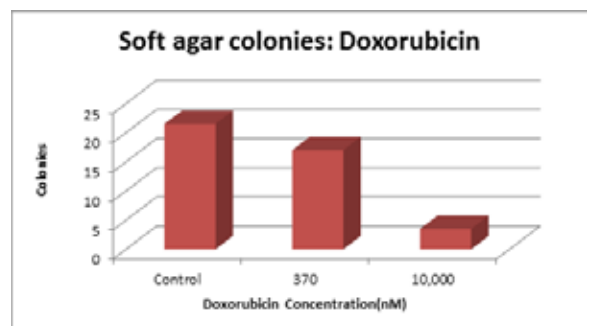

Figure 6. Soft agar assay for Doxorubicin.

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