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Cytotoxicity of Platinum Anticancer Drugs in Mammalian Cell Lines of Metastatic Cancer

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CYTOTOXICITY OF PLATINUM ANTICANCER DRUGS IN MAMMALIAN CELL LINES OF METASTATIC CANCER

A Capstone Project Presented in Partial Fulfillment of the Requirements for the Degree Bachelor of Science with Honors College Graduate Distinction at Western Kentucky University

By
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May 2017

*****

CE/T Committee:
Professor Blairanne Williams, Chair
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2017
This thesis is dedicated to those who taught me the meaning of research and those who never give up.
ACKNOWLEDGEMENTS

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I would also like to thank my parents, Anthony and Edith Evie, who have helped me come this far, have supported me in my endeavors and given me the opportunity to experience a new place.
Abstract

With the invention of advanced technology, focus has been put on understanding and looking for potential cures for many diseases, one of which is cancer. The difference in the leaving and non-leaving ligands of the FDA approved cancer drugs contributes to the differential cell specific cytotoxic effects. These drugs such as oxaliplatin approved for colorectal cancer, cisplatin approved for testicular cancer, and their analogs were used to treat different cancer cell lines in an MTT assay. This project aims to determine how changing the molecular shape of these compounds affects their uptake and toxicity into different cell lines. The assay used the metabolism of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliuM bromide to its insoluble purple colored formazan. The cytotoxicity of these compounds are measured using the absorbance which decreases with increasing drug concentration to calculate the IC50. This value gives the concentration of compound that inhibits mitochondrial reductase by 50%. The cell lines used in these experiments are the NTERA-2 cells, most notably the prostate cancer cell lines with the 293 Human Embryonic Kidney (HEK), a noncancerous line, cells as the control. The IC50 for the compound Pt (S, S-dach) (ox) was 20 µm. There was no significant increase in its effect on the NTERA-2 cells when the concentration was higher while the compound Pt(Me₂dach) (ox) did not inhibit up to 50% cell survival in the NTERA-2 cells even at a concentration was 100 µm. Pt(en)Cl₂ did not give an IC50 value in the HEK cells (control cells). For the experiments on NTERA-2 cancer cells, Pt-(S, S-dach) (ox) was more effective and cytotoxic than Pt(Me₂dach) (ox).

Keywords: Cytotoxicity, MTT assay, IC50
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SECTION ONE

INTRODUCTION

Many of the drugs present in the market to combat cancer are platinum-based drugs, such as oxaliplatin and cisplatin. Cisplatin was the first anticancer drug to be discovered. It was discovered in 1845 and accepted for use in 1978\(^{(1)}\). It has since then been used for treatment of various tumors including ovarian, non-small cell lung cancers and testicular cancer \(^{(1)}\).

![Cisplatin structure](image)

Figure 1: Cisplatin structure \(^{(2)}\)

Cisplatin works by binding to DNA and inhibiting DNA replication. This works effectively on rapidly dividing including cancer cells. The chloride ligands are termed the leaving ligands because they are replaced by water to form cis-[PtCl(NH\(_3\)]\(_2\)(H\(_2\)O)]\(^+\) inside the cell where the chloride concentration is low \(^{(3)}\). Further displacement of the water with mainly purine bases of DNA cause 1,2- and less commonly 1,3-inter and intrastrand cross linkages (cisplatin’s mode of cytotoxic action is 1,2-intrastrand) \(^{(3)}\). When the cell tries and fails to repair the damage, apoptotic signals are activated, and the cell is destroyed. One of the apoptotic pathways include the Fas/Fas ligand pathway which activates Casp-8 and Casp-3 that lead to apoptosis \(^{(4)}\). Fas is expressed on tumor cells and the binding of the Fas ligand (FasL) leads to the activation of the caspases. Another major pathway involved the activation of p53 which activates the pro-apoptotic Bax protein to cause release of cytochrome c from the mitochondrion. This release of cytochrome C causes apoptosis through Casp-9 and Casp-3 \(^{(4)}\). Casp-8, Casp-3 and Casp-9 are
caspases (cysteine-aspartic proteases) which are enzymes that cut proteins after the aspartic acid amino acid residue (4). These and other mechanisms of apoptosis are given in the diagram below. Other mechanisms include arrest of the cell cycle and inhibition of DNA repair which are mechanisms that occur with all apoptotic deaths. These various mechanisms ensure that if the cell somehow manages to escape one pathway, it will still be targeted and destroyed by other means.

Figure 2: The mechanism for activation of apoptosis (5).
The compounds used in these experiments are Pt(en)Cl₂, Pt(Me₂dach)(ox) and Pt(S,S
dach)(ox), which are analogs of the FDA-approved cancer drugs cisplatin and oxaliplatin.

Oxaliplatin is a compound used to treat colorectal cancer, and it works through a mechanism similar to cisplatin. The difference between the compounds is in the nature of their leaving ligands. oxaliplatin’s leaving ligand is the bidentate 1,2-diaminocyclohexane and Pt(Me₂dach) is a dimethylated form of oxaliplatin’s leaving ligand (6). Pt(en)Cl₂ has the monodentate chloride ligands like cisplatin while Pt (S,S dach)(ox) has a bidentate ligand like oxaliplatin. Pt (S, S dach) (ox) is the enantiomer of oxaliplatin with its S, S- configuration instead of the R, R- configuration. Figures 3 and 4 show a comparison between the compounds oxaliplatin, Pt(en)(ox), Pt (S, S dach) (ox), and Pt(Me₂dach) (ox) which is also called (R, R)-N, N’-dimethyl-1,2-diaminocyclohexaneplatinum oxalate.

![Figure 3: Oxaliplatin structure (7)](image-url)
Figure 4: Novel platinum compounds used in the experiments with Pt(en)(ox).

The cell line used in the experiments were the NTERA-2, which are metastasized carcinoma cells derived from a human testis. The control cells for the experiments are the non-cancerous Human Embryonic Kidney 293 (HEK 293) cells. These cell lines are compared to measure the toxicity of the compounds on healthy cells. The HEK 293 cells were created by a transformation with sheared adenovirus 5 DNA that is suspected to have preferably targeted the neuronal lineage cells present in the kidney of an aborted fetus (8). Our focus in these experiments is to measure the percent survival of the cell lines in response to the novel compounds that are analogs of already approved platinum drugs. The novel compounds are synthesized by our collaborator and have not been previously tested for cytotoxicity. Comparisons of cytotoxicity are made by determining the IC50 (50% Inhibitory concentration) value after exposing the cells to the compounds at differing concentrations. The IC50 is the concentration of compound at which half the mitochondrial reductase enzyme activity is inhibited. This inhibition only occurs in the living cells because they have active mitochondrial reductase.
The MTT salt is a yellow tetrazolium dye also known as MTT 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide which is metabolized by the mitochondrial reductase in the mitochondria of the living cells to form insoluble formazan precipitate which has a purple color (1).

![Diagram of MTT salt conversion](image)

Figure 5: The conversion of MTT salt to formazan by mitochondrial reductase occurs only in living cells (9)

The cells are solubilized using dimethyl sulfoxide (DMSO) so that the color is released into the solution. The assay demonstrates the mitochondrial function of the live cells by measuring the activity of the mitochondrial enzyme; therefore, absorbance values are a measure of the number of live cells present. The more cells are present, the brighter the purple color and the higher the absorbance values. Absorbance values for each concentration of compound are compared with those of the control or unexposed wells, and the values are normalized. The percent survival values and these values are plotted on a graph against the corresponding compound concentration to determine the IC50. Using new platinum compounds, the effect of molecular structure on the survival of the NTERA-2 cell line is investigated as a measure of their cytotoxicity.
SECTION TWO

METHODS

Tissue Culture

The cells are cultured in Dulbecco’s Modified Eagle Medium (DMEM) media containing 10% Fetal Bovine serum (FBS) or FBS Essence (VWR Life Science) as standard culture media. Cells grown in a water-jacketed incubator at 37 degrees Celsius and 5% CO₂. All lines used in these experiments are adherent and attach to the culture dishes. Standard cell culture protocol involves subculturing the cells once they have reached a confluency of 80-90% using 0.25% trypsin to lift the cells off the plate and give the cells more space for division.

To subculture, warm up DMEM and trypsin to 37 degrees before use. Remove the old media from the dish and wash with Phosphate Buffered Saline (PBS). Remove PBS and then add 1mL of 0.25% trypsin and incubate at 37 degrees Celsius and 5% CO₂ for no more than 10 minutes. Next, add 4mL of fresh media containing FBS or FBS essence to the plate and using a pipette mix the solution until the clumps of cells are separated as seen under a microscope. Afterwards, transfer the volume of cell solution needed to another plate, dilute to 10mL with media and incubate at 37 degrees Celsius and 5% CO₂.

Cytotoxicity Assay

To set up the cytotoxicity assay, cells are subdivided and counted for accurate plating. After the solution is mixed to break up cell clumps per the above protocol, 10μL is pipetted into each side of a hemocytometer and the number of cells in the 1mm by 1mm squares are counted. At least four squares across both sides are counted and the result is averaged with a minimal number of 100 cells counted on each side of the hemocytometer. For plating, this value is
multiplied by $10^4$ cells to give the concentration of cells. The cells were plated at 500,000 cells for each dish. 500,000 cells are divided by the concentration determined by the hemocytometer to give the volume of cell solution. The mixture is then brought up to 13ml final volume with media. After diluting, 500µL of the mixture is pipetted into each of the 24 wells of the plate which is incubated afterwards at 37degrees Celsius and 5% CO₂. Each experiment consists of two 24 well plates to give an n value of 1.

The plates are incubated for 48 hours to ensure the cells adhere to the wells and then the media is removed and replaced with 250µL of platinum compound of different concentrations into each well. Each concentration is pipetted in three wells (triplicate). An untreated control is created in triplicate with 250µL of fresh media pipetted onto the cells instead of platinum compound. The plates are incubated for 24 hours after which the compound is removed and 450µL MTT salt solution is pipetted into each of the wells and incubated for 3 hours. The MTT salt solution consists of 400µl media and 50µl MTT salt for each well. After 3 hours, MTT salt solution is replaced with 200 µL DMSO solution in each well and incubated for 5 minutes. DMSO solution is made up of 200 µL DMSO and 20 µL Sorenson’s buffer for each well. Sorenson’s buffer is made using 0.133M Na₂HPO₄ and 0.133M KH₂PO₄ in the following step:

Mix 71.5 mL of 0.133M Na₂HPO₄ and 28.5 mL of 0.133M KH₂PO₄ to obtain pH 7.2

The absorbance values of the wells are then measured, after adding DMSO and Sorenson’s buffer, at 550 nm using a plate reader. The plate reader used is the H1 synergy Microplate Reader in the WKU Biotechnology Center.
SECTION THREE

RESULTS

Pt (S, S-dach) (ox) NTERA-2 cells

The normalized values for the percent survival of Pt (S, S-dach) (ox) and their corresponding compound concentrations are given in the table below.

<table>
<thead>
<tr>
<th>Pt (S,S-dach)(ox)(µM)</th>
<th>Percent Survival(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>76.98956812</td>
</tr>
<tr>
<td>10</td>
<td>57.52290569</td>
</tr>
<tr>
<td>20</td>
<td>48.13844982</td>
</tr>
<tr>
<td>30</td>
<td>43.75039675</td>
</tr>
<tr>
<td>40</td>
<td>37.64997143</td>
</tr>
<tr>
<td>50</td>
<td>36.23965805</td>
</tr>
<tr>
<td>60</td>
<td>32.38589475</td>
</tr>
</tbody>
</table>

Table 1: Percent Survival values for Pt (S, S-dach) (ox)

The graph of this values are given in the figure below.
Figure 6: Graph of survival values for Pt (S, S-dach) (ox), (n=1)

From the graph, the IC50 value was determined to be approximately 20µM for Pt (S, S-dach) (ox). The values also showed small standard deviation denoted by the error bars on the graph. The above data shows the results of one experiment. For this, the average percent survival values for the triplicate wells in both plates used in each experiment were calculated. These results were determined from one experiment equivalent to one n value. Subsequent experiments produced limited toxicity, and we were unable to determine IC50 values. As a means of comparison, the IC50 values and the corresponding graphs for multiple experiments is shown below.
<table>
<thead>
<tr>
<th>Pt ($S,S$-dach)(ox)(µM)</th>
<th>Percent Survival(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>88.9203</td>
</tr>
<tr>
<td>10</td>
<td>59.6547</td>
</tr>
<tr>
<td>20</td>
<td>56.69303</td>
</tr>
<tr>
<td>25</td>
<td>58.29741</td>
</tr>
<tr>
<td>30</td>
<td>61.27593</td>
</tr>
<tr>
<td>50</td>
<td>53.67068</td>
</tr>
<tr>
<td>100</td>
<td>53.66879</td>
</tr>
</tbody>
</table>

Table 2: Percent survival values from subsequent experiments for Pt ($S, S$ -dach) (ox)

The values given in table represent the average of four 24 well plates completed at one time.

Four plates were simultaneously and independently plated using separate dilutions from the same counted cell populations, which gave an n of 1.

![Graph of percent survival for Pt ($S, S$ -dach) (ox) (n=1)](image-url)
The values used for the graph represent the average percent cell survival values of triplicate wells for four different plates, n=4. The slight increase in survival at 30µM is not significantly different from the concentrations close to that value indicating that there is no significant difference in cell survival. The percent survival decreases steeply till about 10µM after which the toxicity levels off. The lowest percent survival value is 53.7% at 100µM, which is not enough cell death to provide an IC50.

Pt(Me2dach) NTERA-2

The percent survival values and the corresponding graph for the experiment on Pt(Me2dach) (ox) is given below, n=1.

<table>
<thead>
<tr>
<th>Pt (Me2dach)(ox)(µM)</th>
<th>Percent Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>58.87697</td>
</tr>
<tr>
<td>40</td>
<td>54.37115</td>
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<tr>
<td>50</td>
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<td>60</td>
<td>48.64004</td>
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<tr>
<td>70</td>
<td>48.81032</td>
</tr>
<tr>
<td>80</td>
<td>49.24994</td>
</tr>
<tr>
<td>120</td>
<td>47.8982</td>
</tr>
</tbody>
</table>

Table 3: Percent survival values for Pt (Me2dach) (ox)
Figure 8: Graph of percent survival values for Pt(Me₂dach) (ox) (n=1)

From these results, all the values had small standard deviation. The above data is graphed alone due to the limited toxicity and different compound concentrations. The IC50 from this graph is at about 60µM. The previous experiments were comparable to that of Pt (S, S-dach) (ox) above which did not give percent survival values below 60%.

**Pt(en)Cl₂ NTERA-2**

For Pt(en)Cl₂, none of the results gave an IC50 value because the percent cell survival did not go below 60%. The results shown below are from an average of two independent experiments after the outliers were removed.
<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Percent Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>87.09514</td>
</tr>
<tr>
<td>25</td>
<td>81.51782</td>
</tr>
<tr>
<td>50</td>
<td>78.33663</td>
</tr>
<tr>
<td>125</td>
<td>72.58836</td>
</tr>
<tr>
<td>150</td>
<td>68.31046</td>
</tr>
<tr>
<td>175</td>
<td>67.97564</td>
</tr>
<tr>
<td>250</td>
<td>62.7451</td>
</tr>
<tr>
<td>300</td>
<td>61.74104</td>
</tr>
<tr>
<td>500</td>
<td>61.8357</td>
</tr>
</tbody>
</table>

Table 4: Percent cell survival values for Pt(en)Cl₂

Figure 9: Graph of NTERA-2 cell percent survival for Pt(en)Cl₂ (n=2)
Pt(en)Cl₂ HEK 293

Pt(en)Cl₂ was the only compound tested in this cell line. The results did not show percent survival below 50% and so an IC50 value was not determined. The average values from two experiments is given in the table below.

<table>
<thead>
<tr>
<th>concentration (µM)</th>
<th>Percent survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>97.76473979</td>
</tr>
<tr>
<td>2.5</td>
<td>87.4768298</td>
</tr>
<tr>
<td>5</td>
<td>79.74935413</td>
</tr>
<tr>
<td>10</td>
<td>81.0812516</td>
</tr>
<tr>
<td>25</td>
<td>70.37111625</td>
</tr>
<tr>
<td>50</td>
<td>61.51303013</td>
</tr>
<tr>
<td>100</td>
<td>59.4215546</td>
</tr>
</tbody>
</table>

Table 5: Percent survival values of HEK 293 cells for Pt(en)Cl₂

The graph of the platinum compound concentration and the corresponding percent cell survival are given in the graph below.
Figure 10: Graph of HEK 293 percent cell survival for Pt(en)Cl₂ (n=2)

From the graph, there was a little range in the values as seen from the error bars and the shape of the curve followed what was previously seen. It dropped sharply before evening out. The HEK 293 is the control cell line, which is compared to the NTERA-2 cell lines. The results for the graph was calculated from the average normalized percent survival values of two individual experiments. Each of those experiments were done on two plates with each concentration in triplicate.
SECTION FOUR
DISCUSSION

The experiments measured the toxicity of the FDA-approved drug analogs: Pt(S,S-dach)(ox), Pt(en)Cl₂ and Pt(Me₂dach)(ox) on NTERA-2 and HEK 293 cell lines. The drugs were expected to be toxic to both of the cell lines because they disrupt the DNA replication process which is important to all the cells (3,4). All the experiments showed toxicity as expected. As seen in the graphs above, the cells died rapidly at first and then cell death reached a plateau. It is possible that reduced uptake of the drug affected its ability to enter the cells and cause cell death. Another possible explanation is that the equipment had reached the detection limit and little differences in cell survival was not recorded. Pt (S, S-dach) (ox), and Pt(Me₂dach) (ox) are analogs of oxaliplatin which has been experimented with HCT116 colon cancer cells to have an IC50 of 15µM (10). This experiment with HCT116 cancer lines used MTS salt and 48 hours platinum exposure compared to MTT salt and 24 hours exposure in my experiment on NTERA-2 cells (10).

From the experiments, both Pt (S, S-dach) (ox) and Pt(Me₂dach) (ox) gave percent survival values less than 50% with their IC50 being 20µM and 60µM respectively. The IC50 of Pt (S, S-dach) (ox) is close to the 15µM of oxaliplatin in HCT116 cells. In hindsight, this corresponds to literature on the effect of chirality on the reactivity of the drugs. Enantiomers are known to have different reactivity with one of them, in most cases, being more reactive than the other (11). Pt(S,S-dach) (ox) is an enantiomer of oxaliplatin and in the experiments, it showed the most toxicity. Oxaliplatin is used for treatment of colorectal cancers, but its enantiomer used here affected testicular cancer cells (6). Pt(Me₂dach) (ox) is also an analog of oxaliplatin and this could have contributed to its cytotoxic effect.
With Pt(en)Cl$_2$ and Pt(Me$_2$dach) (ox), some of the experiments had very low control values, while others had little cell death across the whole plate. The absorbance values for the control in these experiments were so low that they were not much different from the other concentrations. This was an issue because the control values were supposed to have the highest percent survival, ideally 100% cell survival. Some other experiments did not have enough cell death across the plate, in these cases, the survival values for the control and the highest platinum concentrations were not significantly different. Values like these were removed from the data set included in the graphs. Many of the experiments started having problems when they were done after the FBS was changed to FBS essence. FBS is used in cell culture as a supplement for growth because it contains “embryonic growth factors” (12). This change is suspected to be the cause for the broad range and diversity in the results. It was unexpected because a growth curve indicated similar growth in the presence of either FBS or the synthetic essence. Apart from these, no other significant change was made to the protocols and materials used.

More recent experiments had issues with cells coming off the plate after the MTT salt had been placed on it for 3 hours. This affected the absorbance values and the original protocol using 250µL MTT solution was changed to 450µL MTT solution. After the change, 450µL reduced the amount of cell loss and the absorbance values become normal. With the reduced cell death in results from Pt(en)Cl$_2$ and Pt(Me$_2$dach)(ox), there is some speculation of the role of uptake in these results. These compounds may have structures that are preventing them from being transported into the cells therefore reducing the cell death. Further studies have to be done to measure the uptake in the cells.

Another possible factor causing the reduces cell death is the number of cells across the whole plate. 500,000 cells were counted and plated evenly across each 24 well plate. This
number was chosen because previous growth curves showed that it was the optimal number for cell growth. With the low control values and other problems that arose during the experiment, another growth curve will be done using both HEK 293 and NTERA-2 cells to confirm the best cell number for optimum growth.

The number of experiments used to calculate the average and plot the graph was different for each compound. Compounds such as Pt(Me$_2$dach) (ox) had only an n of 1. This was because other independent experiments were not used because of the little cell death. An example of another experiment performed using Pt(Me$_2$dach) (ox) is given in figure 11 in the appendix. The values were not consistent and the lowest cell survival at a concentration of 100µM was at about 92%. This was one of the reasons the data was discarded from the overall calculations. Each of the experimental results that were not added to the final calculations were removed because of low cell death and low control values caused by cell loss during the experiments.

Pt(en)Cl$_2$ was used in the experiment with HEK 293 as the control cell. Comparing the results with that of the NTERA-2 cell shows that they both have comparable reactions to the drug. Pt(en)Cl$_2$ is affecting both the cancerous and non-cancerous cells similarly. To truly be able to compare the control cell lines with NTERA-2 cells, other compounds have to be tested. In this experiment, only Pt(en)Cl$_2$ was used for both cell lines, so it does not give a good estimation of the effects. Each experiment has to be done multiple times to ensure that the results are valid, accurate and representative of the actual effects.

All the compounds used in this experiment are novel ones which have not been used on these cell lines and so each conclusion made is an extrapolation of results from other studies that has to be tested again. Later on, studies can be carried out on other cancer cell lines using these and other platinum analog compounds. Further experiments will start with a cell growth curve
for both cell lines because it is suspected that 500,000 cells may not be the optimum number for cell growth as was gotten from previous growth curves.

One limitation to the experiments is that they use cancer cell lines and involve in vitro experiments rather than cancer cells in an in vivo experiment. This has to be taken into consideration when doing these experiments. Although, the goal is to find the toxicity of the platinum compounds, the cancer cell lines used have been cultured in media for several generations and may have developed mutations that allow them to fare in that environment. Also, the environment of cancer cells in the human body is very different from that of the media. The human body has many more molecules present including cytokines and proteins that may modulate the environment. Therefore, the results gotten may not always translate to the actual effect on a human body. Regardless, this characterization helps in making somewhat accurate estimations of the effects and can be used as a guideline for future translational research on the novel platinum compounds.
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Figure 11: Graph of percent cell survival for Pt(Me2dach) (ox) (n = 1)