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Effects of Alternative Chemotherapeutic Agents on Thyroid Cancer Cell Line MI-1

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**EFFECTS OF ALTERNATIVE CHEMOTHERAPEUTIC AGENTS ON THYROID
CANCER CELL LINE ML-1**

A Master's Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree
Master of Science, Biology

By

Husref Rizvanovic

May 2020

EFFECTS OF ALTERNATIVE CHEMOTHERAPEUTIC AGENTS ON THYROID CANCER CELL LINE ML-1

Biology

Missouri State University, May 2020

Master of Science

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ABSTRACT

Though differentiated thyroid carcinomas have decent prognosis when detected early, radioactive iodine (RAI) resistant and advanced thyroid cancers are still difficult to treat with existing therapies. Better therapeutic agents are needed. Studies have shown that aggressive thyroid cancers (ML-1) express the extracellular matrix protein, matrix metalloproteinase (MMP-2). MMP-2 has been linked to metastasis and aggressiveness of several cancers and has been shown to play a crucial role in tumor invasion. Chlorotoxin is a selective MMP-2 receptor agonist, and Saporin is a well-known ribosome-inactivating protein used for anti-cancer treatment; however, these two agents have never been studied when conjugated together. I hypothesize that Chlorotoxin-conjugated to Saporin (CTX-SAP) would impede the growth of aggressive thyroid cancer cell lines expressing MMP-2 receptors. Moreover, there is also an unmet need for better platinum-based anti-cancer drugs. Cisplatin, an FDA approved anti-cancer drug was tested alongside the novel cyanoximate, Pt (DECO)₂ for its ability to reduce cell viability, reduce superoxide, and increase apoptosis. Results from my study support the potential of CTX-SAP, but offer limited availability for future study. My findings also demonstrate that Pt (DECO)₂ is more effective at reducing cell viability, reactive oxygen species, and increasing apoptosis compared to Cisplatin.

KEYWORDS Saporin, Chlorotoxin, radioactive iodine resistance, thyroid cancer, MMP-2, ML-1, XTT, ROS, apoptosis, Cisplatin

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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INTRODUCTION

Cancer

Cancer poses a threat to human life worldwide, and no one person is exempt from its terrible effects. It is the second leading cause of death in the United States; with recent advancements in medicine, it still takes millions of lives annually (Siegel, Miller et al., 2019). Cancer is characterized as over 100 different types of diseases that can be depicted by uncontrolled cell growth or invasion. These cells originate from healthy cells found in the body that have undergone a genetic transformation that leads to uncontrolled proliferation, and eventually tumor growth (Fouad and Aanei, 2017). These tumors are what we envision when thinking about cancer symptoms and disease progression.

Thyroid Cancer

Thyroid cancer is the most common endocrine malignancy worldwide and has seen growing incidence over the last decade (Grimm, 2017; Rahib, Smith et al., 2014). Estimated incidence of thyroid cancer in the United States for 2020 is 52,890 (Morris, Sikora et al., 2013). It is predicted that by the year 2030, thyroid cancer will be the fourth leading cause of new cancer diagnoses in the United States, replacing that of colorectal cancer (Rahib, Smith et al., 2014). According to the American Thyroid Association, an estimated 20 million Americans have some form of thyroid disease, and of that population, 60% are unaware of their condition (American Thyroid Association, <https://www.thyroid.org/media-main/press-room/>). The American Cancer Society distinguishes four different types of thyroid cancers (Table 1), with two distinct epithelial-cell origins. Papillary Thyroid Cancers (PTC), Follicular Thyroid Cancers

(FTC), and Anaplastic Thyroid Cancers (ATC) are all derived from the follicular cells found in the thyroid epithelium, whereas Medullary Thyroid Cancers (MTC) are derived from the parafollicular or C-cells. Papillary thyroid cancers account for about 80% of all thyroid cancers, making them the most abundant form of thyroid cancer, while also being the least aggressive type. Follicular thyroid cancers account for about 20% of thyroid cancers, and Anaplastic thyroid cancers account for about 2% of all thyroid cancers and tend to be the rarest form of thyroid cancer. Lastly, Medullary thyroid cancers account for about 4% of all thyroid cancers and can also be familial. The remaining ~4% of thyroid cancer cases are due to Hurthle cell thyroid cancers or other rare forms of thyroid cancer. Most thyroid cancers respond well to surgical resection, radioactive iodine, thyroid stimulating hormone (TSH) suppression, tyrosine kinase inhibitors, or external beam radiation (Table 2). However, a subset of these thyroid cancers will develop metastasis and become resistant to radioactive iodine (RAI) treatment (Hirshfield, Aisner et al., 2017), or incur a genetic mutation (Cohen and Lippard, 2001), making them harder to remove. Even with newer therapeutic agents like tyrosine kinase inhibitors, it is very difficult to prevent progression of RAI-resistant metastatic thyroid cancer. Despite advancements in diagnosis and treatment, there is an unmet need for better therapeutic agents in this scenario for the treatment of thyroid cancer, and more specifically for the treatment of RAI-resistant, or dedifferentiated thyroid cancers.

Cell line

ML-1 (ACC-464), thyroid cancer cell line is derived from a dedifferentiated recurrent follicular thyroid carcinoma from a 50-year-old patient. ML-1 is tumorigenic in rodents and has been found to express matrix metalloproteinase-2 (MMP-2), which are extracellular matrix

proteins associated with remodeling and growth factor signaling in healthy cells (Noel, Gutierrez-Fernandez et al., 2012). MMP genes also display a high conservation in regards to their modular structure, and have also been known to play a crucial role in tumor invasion of several cancers (Chakraborti, Mandal et al., 2003). In many cancers, including thyroid, it has been found that MMP-2 is upregulated by the cancer cells; therefore, MMP-2 makes for an ideal protein for target-specific therapy (Grosse, Warnke et al., 2013; Cho Mar, Eimoto et al., 2006).

Current Therapy for Thyroid Cancer

Treatment options for thyroid cancer vary depending on the type of cancer (histology), disease stage and patient characteristics as seen in Table 2. At present, traditional therapies include the use of surgery (thyroidectomy), radioactive iodine, targeted therapies such as tyrosine kinase inhibitors or chemotherapeutic drugs, and external beam radiation. For those individuals that do not wish to completely remove their thyroid, or for cancer in a less progressed state, they can opt for a partial removal of their thyroid, known as a thyroid lobectomy (Sephton, 2019). Additionally, when surgical intervention is required, removal of the lymph nodes in the neck can be necessary to test for cancerous cells as well.

Radioactive iodine treatment, alongside surgical resection or combination therapies has proven to be the most effective solution to treat thyroid cancer. The purpose of RAI therapy is to completely obliterate any remaining thyroid tissue in the body. However, there are still thyroid cancers that no longer uptake iodine (RAI resistant/refractory) and do not respond to RAI, so there is a need for other, better-targeted treatments for these thyroid cancers.

For cancers resistant to RAI therapy, external beam radiation, chemotherapy, and tyrosine kinase inhibitors are typically the alternatives for treatment. The use of targeted systematic

therapies represents major advances for patients with RAI-resistant differentiated thyroid cancers, that until recently, had only few treatment options (Worden, 2014). Currently, the Mayo Clinic and the American Cancer Society recommend clinical trials for patients with advanced RAI-resistant thyroid cancer (<https://www.cancer.org/cancer/thyroid-cancer/treating/by-stage.html>) leading to the need for development of other potential chemical agents that can be used for treatment.

Alternative Chemotherapeutic Drugs

Chlorotoxin (CTX) is a 36-amino acid peptide derived from the venom of the death stalker scorpion, *Leiurus quinquestriatus* (Dardevet, Rani et al., 2015). It was originally used as a pharmacologic tool in studying chloride channels in glioma-specific currents but has gained popularity in cancer research upon the discovery that it possesses targeting properties in many different cancers (Dardevet, Rani et al., 2015). Specifically, it has been shown to bind selectively to isoform 2 of matrix metalloproteinase (MMP-2) (Deshane, Garner et al., 2003). MMP-2 is overexpressed in aggressive thyroid cancers when compared to normal thyroid tissue (Cho Mar, Eimoto et al., 2006). Aggressive follicular thyroid cancers, like ML-1, were also found to overexpress MMP-2 (Cho Mar, Eimoto et al., 2006).

Saporin (SAP), also known as Saporin-S6, is a 30 kDa protein derived from a class of ribosome inactivating proteins originating from the seeds of the Soapwort plant *Saponaria officinalis* (Polito, Bortolotti et al., 2013). The mechanism of action for Saporin has been well studied and has been successfully employed in the creation of many immunotoxins. Like most ribosome-inactivating proteins, Saporin contains enzymes that depurinate rRNAs resulting in the inhibition of protein synthesis (Polito, Bortolotti et al., 2013; Stirpe and Battelli, 2006). Saporin

makes for a great candidate in immunological studies as it is very stable *in vivo* and is resistant to proteases in the blood (Polito, Bortolotti et al., 2013). Since Saporin works through many different cell death pathways, it is hard to develop resistance to it (Polito, Bortolotti et al., 2013). Saporin by itself is unable to cause significant cell damage, as it is not efficient in passively entering or being endocytosed into cells. However, when conjugated with antibodies or other toxins that promote its internalization, it confers lethality as these conjugates have apoptotic and necrotic properties (Stirpe and Battelli, 2006). The first study using an antibody conjugated with SAP was conducted in humans for refractory Hodgkin's disease (Falini, Bolognesi et al., 1992). Seventy-five percent of the patients achieved complete remission and fifty percent experienced relief from symptoms (Falini, Bolognesi et al., 1992). A recent study used Substance P conjugated with Saporin intrathecally in cancer patients with intractable pain to confirm that Saporin can safely and effectively be used at low doses (Frankel, Nymeyer et al., 2014).

Cisplatin (cis-diamminedichloroplatinum, DDP) is a square planar compound with a molecular weight of 300.1 g/mol, and is among the most effective and widely used chemotherapeutic agents for treatment of solid tumors today. In 1978, it became the first FDA-approved platinum compound for cancer treatment (Dasari and Tchounwou, 2014). It is an effective inducer of apoptosis and cell cycle arrest in most cancer cell types as it forms inter- and intra-strand adducts with DNA, and leads to crosslinking of purine bases (Cohen and Lippard, 2001). Its effects have been studied in lung, ovarian, testicular, and head and neck cancers. However, cancer cells can develop resistance to Cisplatin over time, which leads to the rationale behind studying alternative platinum-based chemical agents. This resistance is typically attributed to: 1) reduced intracellular cisplatin accumulation due to poor drug transport or endocytosis; 2) an elevated drug detox system attributed to increased levels of intracellular

proteins like metallothioneins or glutathione; 3) alteration in DNA repair mechanisms that include enforced base excision repair or mismatch repair systems; 4) changes in DNA damage tolerance; and 5) changes in the cancer cells apoptotic cell death pathways or cell cycle arrests (Dasari and Tchounwou, 2014).

Platinum-Derived Pt (DECO)₂ and Pd (DECO)₂

Platinum 2-cyano-2-oximino-*N,N'*-diethylaminoacetamide, or Pt (DECO)₂ and Palladium 2-cyano-2-oximino-*N,N'*-diethylaminoacetamide, or Pd (DECO)₂ are platinum-derived cyanoximates. A cyanoxime is simply a biologically active organic molecule that binds to metal ions via nitrosation reactions. They work as excellent ligands and also as carbonyl reductase inhibitors. They have since been understudied as an alternative to pre-existing metal-containing chemotherapy anti-cancer drugs, such as Cisplatin. Platinum containing anti-cancer drugs are prescribed regularly for treatment of cancer patients. Even though they are very effective, their use is limited due to their high toxicity, poor water solubility, and other several adverse effects associated with heavy-metal drugs, such as organ failure or metal toxicity in human systems (Oun, Moussa et al., 2018; Eddings, Barnes et al., 2004). Nevertheless, there is still a great need for further research in different metal-based anti-cancer drugs as tumors consistently develop drug tolerance over time.

Consequences of Treatment

Like with most other diseases, treatment is never 100% without consequence or side-effect profiles. Current consequences of undergoing thyroidectomy or thyroid lobectomy include damage to the parathyroid, which can result in reduced levels of calcium circulating in the body

(Chahardahmasumi, Salehidoost et al., 2019). Aside from that, surgery also runs the risk of bleeding as well as infection, and ultimately can result in temporary to permanent nerve damage to the vocal cords that could result in difficulty breathing and speaking (Chahardahmasumi, Salehidoost et al., 2019). External beam radiation therapy, or EBRT, can also consequently cause cancer development in local tissue in the future. This is due largely due to the ionizing radiation causing damage to DNA allowing for those genetic transformations as discussed earlier.

Hypothesis

I hypothesize that with less evasive, alternative chemotherapeutic agents, such as the ones introduced in this study, elimination of thyroid cancer may be more efficient, circumventing the need for more invasive therapies.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM; #10-017-CV), fetal bovine serum (#35-010-CV), Penicillin and Streptomycin antibiotics (# 30-002-CI), 75cm² and 25cm²(#430639, #430641U, respectively) culture flasks (were ordered through Corning (New York, USA).

Annexin V-APC (#A35110), Propidium Iodide (#P1304MP), Phosphate Buffered Saline (PBS; # AM9624), Trypsin (#15050057), and Trypsin-EDTA (#25200056) were ordered through Thermo Fisher (USA).

XTT assay and reagents were received from Biotium (Fremont, CA, #3007).

All platinum-based toxins, Cisplatin, Pt (DECO)₂, Pd (DECO)₂ were acquired from Dr. Nikolay Gerasimchuk in the Missouri State Chemistry Department in Springfield, Missouri.

Cell Culture

The cell line used in these studies was ML-1 (ACC-464), which was derived from a stage 4, dedifferentiated follicular thyroid carcinoma from the DSMZ German Leibniz Institute of Microorganisms and Cell Cultures ([www.https://www.dsmz.de/collection/catalogue/details/culture/ACC-464](https://www.dsmz.de/collection/catalogue/details/culture/ACC-464)). Cells were authenticated by the DSMZ and Genetica DNA Laboratories (Burlington, NC) and cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin antibiotics. Cultures were maintained at 37°C and 5% CO₂ in a cell-culture treated 75 cm³ corning culture flask until optimal confluency was reached (75-85%), or stored in liquid nitrogen for long term storage. This cell line was of interest

for this study as it has been known to express MMP-2, which made it a great candidate for testing target-specific chemicals in the ML-1 cells, which are a well-progressed dedifferentiated cell line. All research was approved by the Missouri State University Biosafety committee and institutional review board (IRB) approval was not required.

Toxins

The Chlorotoxin-Saporin (Beta 010) conjugate was acquired from Advanced Targeting Systems (San Diego, CA). This toxin consisted of biotinylated Chlorotoxin bound to a secondary conjugate of Streptavidin-ZAP containing Saporin. Unconjugated Saporin and Chlorotoxin were acquired through Sigma-Aldrich (St. Louis, MO) and solubilized in DMEM.

Conjugated Toxins

After allowing cells 24 hours to incubate and attach to their respective wells (10,000 cells/well on 96-well plate), 2 μ M stocks of Chlorotoxin, Saporin, and Chlorotoxin-Saporin (CTX-SAP) treatments were prepared ([www.https://www.atsbio.com/catalog/data/beta010.pdf](https://www.atsbio.com/catalog/data/beta010.pdf)).

Platinum-Derived Toxins and Solubility Tests

It has been shown that Cisplatin, as well as Pt and Pd (DECO)₂ can only be solubilized in organic solvents such as Dimethyl Sulfoxide, or DMSO (Eddings, Barnes et al., 2004). However, what had not yet been studied was the amount/percent of DMSO needed to solubilize these compounds per mg of chemical agent that wouldn't reduce viability of thyroid cancer cells (Table 3). Therefore, a viability test was conducted as seen in Table 3 to determine what percent of DMSO was needed to solubilize each toxin without causing toxicity starting at 100% DMSO

and then making lower dilutions in DMEM. Based on results from this pilot study, it was determined that a 2% DMSO concentration in DMEM could serve as a negative control since this concentration did not cause a significant reduction in viability in those groups (Figure 1). It was also determined that this concentration of DMSO was sufficient to solubilize the toxins. These tests were done by treating wells seeded with 10,000 cells/well with varying concentrations of DMSO and performing an XTT cell viability assay.

XTT Cell Viability Assay

XTT (2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt) is a colorimetric, reduction-based proliferation assay that quantifies cellular proliferation and viability based on the cleavage of the tetrazolium salt, XTT (<https://www.atcc.org/~media/Attachments/5/6/3/7/16747.ashx>). After 24 hours of incubation with chemical agents, 25 µL of activated XTT dye was added into each triplicate well of 7,500-10,000 cells (without removing media) on the 96-well plate according to the Biotium manufacturer's guidelines ([www.https://biotium.com/wp-content/uploads/2013/07/PI-30007.pdf](https://www.biotium.com/wp-content/uploads/2013/07/PI-30007.pdf)) and quantified using a multi-well spectrophotometer (BioTek ELx808) set to Delta (A450-A630) using a Gen5 plate reading software (number of experimental replicates, n=3). Absorbance at hours 0-7 with XTT dye was recorded and quantified to determine the levels of reduction in viable cells (Aslantürk, 2017).

Native Gel

To test if Chlorotoxin and Saporin were successfully conjugated together, the sample was loaded on a non-denaturing native polyacrylamide gel and band patterns were compared with a

standard ladder (BIO-RAD, USA). If samples were unconjugated, an 85 kDa protein band of SAP-Strep would be visible, and a 5 kDa band would be expected for Biotinylated CTX. Samples were stained with Coomassie Blue and imaged using an Azure c300 gel imager.

Reactive Oxygen Species

In order to measure levels of superoxide production, dihydroethidium (DHE) was utilized in this experiment (Biotium; Fremont, CA). DHE was dissolved in DMSO, and a 10 mM stock was made. On the day of the experiment, a 10 μ M working solution was made in PBS. A 24-well plate was seeded at a density of 50,000 cells/well and incubated for 24 hours (n=3). Wells were then treated with 1.0 mM Cisplatin and Pt (DECO)₂ in 2% DMSO for 24 hours. After harvesting cells in each well, pellets were resuspended in 1 mL of the 10 μ M DHE working solution and incubated for 30 minutes in the dark. After incubation, cells were analyzed with an Attune NxT flow cytometer (ThermoFisher, USA). Excitation and emission wavelengths of DHE were 518nm and 606nm, respectively.

Programmed Cell Death Measurements

Programmed cell death, or apoptosis, was measured using an Attune NxT flow cytometer (ThermoFisher, USA) following the manufacturer's protocol. Using a 24-well plate, 50,000 cells/well were seeded and incubated for 24 hours on a 24-well plate (n=3). After incubation, wells were treated with 1.0 mM concentrations of Cisplatin or Pt (DECO)₂ for 24 hours. After incubation, wells were harvested and resuspend with 1X Annexin V binding buffer ([www.http://cshprotocols.cshlp.org/content/2016/11/pdb.rec088443.short](http://cshprotocols.cshlp.org/content/2016/11/pdb.rec088443.short)) containing Annexin V-APC and propidium iodide based on manufacturer's guidelines (BD Biosciences, USA). Cells

were incubated with these dyes for 15 minutes and analyzed with flow cytometry. Annexin V-APC has an excitation wavelength of 650 nm and PI has an excitation wavelength of 617 nm.

Statistical Analysis

Prism8 statistical software (GraphPad Software Inc.) was used to conduct statistical analyses. One-Way ANOVA was performed assuming Gaussian distribution to compare absorbance values between toxin-treated groups and non-treated controls. Post hoc comparisons were done running Dunnett's test to compare values from toxin-treated groups to the non-treated control (NTC). All values are expressed as the mean and standard deviation of recorded values in triplicate. N refers to the number of plates included in each experiment. P-values calculated from Dunnett's test were adjusted to account for multiple comparisons.

RESULTS

Cancer Cell Viability Tests

To assess if a dose-dependent reduction of cell viability was present, ML-1 cells were treated with conjugated Chlorotoxin-Saporin at concentrations ranging from 0 to 200 nM for 72 hours (Figure 2A). There was an overall statistically significant difference in cell viability at 7 hours of incubation with XTT and PMS after 7 hours of treatment ($F=4.286$, $p=0.0057$) with an apparent dose-dependent trend for decreased cell viability with increasing concentration of the conjugate. Post hoc statistical comparisons using Dunnett's test showed no significantly reduced viability for cells exposed to 2, 10, or 20 nM relative to non-treated control ($p>0.05$). However, cells exposed to CTX-SAP conjugate at concentrations of 40, 100, and 200 nM had significantly reduced viability relative to non-treated controls (Dunnett's tests, $p=0.0138$, $p=0.0052$, and $p=0.0037$ for 40, 100 and 200 nM, respectively, relative to non-treated control (NTC)).

I repeated the experiment with a higher concentration of the CTX-SAP conjugate and once again assessed viability at 7 hours of XTT incubation (Figure 2B). I used concentrations of 2, 20, and 600 nM, and there was a statistically significant difference in cell viability assessed with a one-way ANOVA ($F=44.24$, $p<0.0001$). Post hoc comparisons using Dunnett's test revealed that viability was significantly reduced for the 600 nM group relative to the non-treated control (difference in absorbance of 0.8410, $p<0.0001$). However, the lower concentrations of the conjugate did not significantly differ from the non-treated control ($p=0.2492$ and $p=0.5658$ for 2 and 20 nM, respectively).

To determine if unconjugated Chlorotoxin exhibits cytotoxicity without Saporin, ML-1 cancer cells were treated with unconjugated Chlorotoxin at concentrations that ranged from 0 to

600 nM for 72 hours. In Figure 2C, after 72 hours of chemical treatment, there was not an overall statistically significant difference in cell viability assessed after 7 hours of incubation in the presence of XTT and PMS ($p>0.05$). The largest difference was slightly increased viability of the cells exposed to 600 nM of unconjugated CTX relative to the non-treated control (mean difference in absorbance -0.054). However, this was not statistically significant with a Dunnett's test ($p=0.1279$).

To establish if unconjugated Saporin exhibits cytotoxicity without Chlorotoxin, ML-1 cancer cells were exposed to unconjugated Saporin at concentrations that ranged from 0 to 600 nM (Figure 2D). There was not an overall statistically significant difference in cell viability after 7 hours of incubation with XTT and PMS ($p>0.05$). The largest difference was improved viability (absorbance) of the cells exposed to 20 nM of unconjugated SAP relative to the non-treated control (mean difference in absorbance -0.05325). However, this was not statistically significant with a Dunnett's test ($p=0.0874$).

In these novel experiments, ML-1 cancer cells were exposed to either cisplatin or the novel cyanoximate, Pt (DECO)₂, at concentrations ranging from 0 to 1.0 mM for 24 hours (Figure 3) to assess if Pt (DECO)₂ exhibits cytotoxicity similar the level observed with Cisplatin. There was an overall statistically significant reduction in cell viability after 7 hours of incubation of activated XTT dye ($F=45.77$, $p<0.0001$). There was an apparent dose-dependent reduction in cell viability with increasing concentration of Cisplatin. Post hoc statistical comparisons using Dunnett's test showed significant reduction at 1.0 and 0.1 mM of cisplatin treatment compared to the non-treated control (Dunnett's tests, $p<0.0001$, and $p=0.001$, receptively). Statistical comparisons of 1.0 mM of Pt (DECO)₂ with non-treated control showed a significant reduction in cell viability greater than what was seen by cisplatin at the same concentration ($P<0.0001$).

However, at lesser concentrations of Pt (DECO)₂, no significant reduction of cell viability was observed (0.1 mM, p=0.9999; 0.01 mM, p=0.9994). Both chemicals were dissolved in 2% DMSO, which displayed no significant reduction in cell viability when compared with the non-treated control (p=0.4376).

Native Gel

To test for successful conjugation of Chlorotoxin-Saporin, protein samples were resolved by a non-denaturing native gel and band patterns were observed (Figure 4). Due to the nature of the patterns, it was evident that the two chemicals had been conjugated; however not properly. Based on the molecular weights of the protein standards included in the ladder, it was clear that multimeric complexes had been formed according to the molecular weights present when compared to the ladder and the weight of each individual chemical.

Platinum-Based Reduction of Superoxide

Accumulation of superoxide levels (DHE) were measured with flow cytometry to assess changes in oxidative stress before and after treatment. Percentages of superoxide detection utilizing the marker DHE are shown in Figure 5. At concentrations of 1.0 mM Cisplatin and Pt (DECO)₂, it was observed that levels of superoxide are significantly reduced compared to that of the control (F= 28.57, p=0.0001). At 1.0 mM concentrations of Cisplatin, levels of superoxide were reduced by 51.24% when compared with the non-treated control (p=0.0006). Wells treated with 1.0 mM Pt (DECO)₂ displayed a significant reduction at 46.89% (p=0.0003). Non-treated wells and wells treated with 2% DMSO displayed the highest percent averages of superoxide production (76.75% and 75.27%, respectively; Dunnett's test, p=0.9884).

Increase of Apoptosis

Changes in apoptotic levels due to treatment are seen in Figure 6. After 24 hours of treatment, it was evident that both 1.0 mM of Cisplatin and Pt (DECO)₂ treatment increased the levels of programmed cell death (Figures 6 B,D, F, & H), compared to non-treated control (NTC). In regard to efficacy in causing cell death, neither treatment caused increase in necrosis (Figure 6A & E; $F=1.506$, $p=0.2854$). However, we observed a significant increase in late apoptosis in cells treated with 1.0 mM Cisplatin and Pt (DECO)₂ ($F<0.0001$; $p=0.0025$, $p<0.0001$, respectively) (Figure 6F). Moreover, there was a four-fold increase of late apoptosis with Pt(DECO)₂ observed, suggesting more efficient and quicker induction of cell death (Figure 6 B & F). There was a 250% increase in early apoptosis upon treatment with 1.0 mM Cisplatin compared to the control (Figure 6 D & H; $F=16.45$, $p=0.0019$).

To assess specificity of Cisplatin and Pt (DECO)₂ on non-cancerous cells, mouse-derived fibroblasts were treated with 1.0 mM Cisplatin or Pt (DECO)₂ for 24 hours to assess changes, if any, to cell death. Results shown in Figure 7 demonstrate that both drugs kill cells present with no indication of specificity to killing cancer cells over non-cancer cells. An observed increase in late apoptosis and early apoptosis was seen, with no increase in necrosis (Figure 7A & E). The significant increase in late apoptosis due to Pt (DECO)₂ treatment suggests swifter cell death (Figure 7 B & F; $F=378.9$, $P<0.0001$) to the cells. Additionally, viability was reduced significantly in both treatment groups ($F=130.5$, $P<0.0001$).

DISCUSSION

Effectiveness of CTX-SAP

The first part of my research addressed the effectiveness of the potential chemotherapeutic benefit of a novel chemical conjugate. Biotinylated Chlorotoxin bonded to the secondary conjugate Streptavidin-ZAP containing Saporin holds promise of being used as an alternative chemotherapeutic agent. Results suggest that the conjugate effectively inhibited proliferation or viability of the ML-1 thyroid cancer cells in a dose-dependent manner *in vitro* (Figure 2C and 2D). Saporin, a plant toxin derived from the seed of *Saponaria officinalis*, aided as an effective ribosome-inactivating protein to inhibit protein synthesis of the cancer cells in the present study and in similar studies (Giansanti, Flavell et al., 2018; Bergamaschi, Perfetti et al., 1996). Moreover, Chlorotoxin, a 36 amino-acid peptide from the venom of the deathstalker scorpion, *Leiurus quinquestriatus*, has proven an effective vehicle for anti-cancer drug delivery (Ojeda, Wang et al., 2016). However, my data provides evidence that the two agents need to be bound together in order to confer any cytotoxic effect. Results shown in Figure 2A and 1B demonstrate a lack of reduction of cancer cell viability upon unconjugated treatment. These results are in agreement with previous reports that Saporin needs a vehicle to properly confer lethality (Polito, Bortolotti et al., 2013). Fortunately, Chlorotoxin is a well-studied matrix metalloproteinase isoform 2 (MMP-2) agonist, making it an effective vehicle for internalization (Deshane, Garner et al., 2003). MMPs are extracellular matrix proteins associated with remodeling and growth factor signaling within the cell (Deshane, Garner et al., 2003; Itoh, Tanioka et al., 1998). MMPs are known to be upregulated in many cancers as they are implicated

in tumor growth, angiogenesis, invasion, and inflammation (Itoh, Tanioka et al., 1998).

Activation of MMP-2 can occur at the cell membrane and is enhanced in many different cancers, including thyroid cancers (Nakamura, Ueno et al., 1999; Aust, Hofman et al., 1997; Hofmann, Laue et al., 1998; Han, Dugas-Ford et al., 2015). Previous studies have shown that Chlorotoxin promotes endocytosis of MMP-2, which is directly involved with the invasive potential in ML-1 cells (Kalhori and Törnquist, 2015).

Presently, a significant dose-dependent inhibition of ML-1 thyroid cancer cell viability is conferred upon treated cancer cells with no apparent effect upon treatment of unconjugated drug (Figure 2). Further research needs to be done to elucidate the exact mechanism behind internalization of Chlorotoxin conjugates into the cell. Research findings support the notion that internalization occurs via receptor-mediated endocytosis of MMP-2 with MMP-14 (Han, Dugas-Ford et al., 2015). Additionally, if resources were not limited with the manufacturer, more research needs to be done to determine the effect of conjugate treatment on oxidative stress, cell death, and specificity in non-cancerous cell lines. In my study, conjugating Biotinylated-Chlorotoxin with just Streptavidin-Saporin (excluding ZAP) did not cause a significant reduction in viability of cancer cells treated with 1.2 μ M of CTX-SAP. Based on results from the non-denaturing native gel (Figure 4 and 8), multimeric complexes formed, thus suggesting a hinderance in receptor-mediated endocytosis, ultimately mitigating the effect of the conjugate. If conjugated successfully, we would have expected to have seen a band present around 90 kDa representing the binding of one biotinylated Chlorotoxin to one Streptavidin-bound Saporin. The next step after either manufacturing of CTX-SAP Beta010 is revisited or effective conjugation of ZAP domain is accomplished would be to conduct *in vivo* tests to assess efficacy in physiological conditions on healthy and cancer-induced mice.

Assessment of Pt (DECO)₂

At the start of this study, little was known about cyanoxime solubility and sensitivity to thyroid cancer cells. Therefore, testing needed to be conducted to assess proper solubility of cyanoximes in DMSO (Table 3) and the concentration of DMSO (Figure 1 and Figure 9) necessary for solubilization that didn't confer significant cytotoxicity *in vitro*. This was realized upon the first XTT assay of Cisplatin, Pt (DECO)₂ and Pd (DECO)₂ at concentrations ranging 0-1.0 mM in 10% DMSO (Figure 9). Results in Figure 9 suggested drastic effects of each treatment group to cells; however, this did not account for the drastic cytotoxic effect due to DMSO alone. Therefore, ML-1 cells were treated with just DMSO at varying concentrations to assess the threshold at which DMSO did not confer significant reduction on viability (Figure 1), as well as what concentration of DMSO necessary to fully solubilize each chemical (Table 3). Dr. Gerasimchuk in the Eddings, Barnes et al., paper solubilized the toxins in 100 mM DMSO aliquots and diluted them down to 1.0 mM for treatment (2004). Due to limited availability of chemicals, Pd (DECO)₂ was not able to be considered for more studies as it required a higher concentration of DMSO than did Cisplatin or Pt (DECO)₂. Therefore, a final concentration of 2% DMSO was decided to solubilize Cisplatin and Pt (DECO)₂ to create 50 mM stocks for each test.

Cyanoximate Comparisons

Platinum complexes have been widely used as anticancer agents since the accidental discovery of cisplatin as a potent anticancer treatment in 1965 (Dasari and Tchounwou, 2014; Bergamo and Savi, 2015). Not approved by the FDA until 1978, Cisplatin and its analogs remain the staple treatment for combating many cancers (Dasari and Tchounwou, 2014). Cisplatin,

Carboplatin, and Oxaliplatin share similar mechanisms of action to confer toxicities, and therefore, also share similar side effect profiles (Ndagi, Mhlongo et al., 2017). These side effects include weakening the immune system in patients, anemia, myelosuppression, and organ/tissue damage that can lead to nerve damage and hearing loss (Dannen, Cornelison et al., 2020). Moreover, cancer cells can transform to acquire a resistance to Cisplatin treatment; therefore, there is an unmet need to develop newer platinum-based anticancer drugs (Dasari and Tchounwou, 2014; Ndagi, Mhlongo et al., 2017). The present study addressed the effectiveness of a novel platinum-based drug, Pt (DECO)₂, on its potential use as an anti-cancer drug, and its potential for reducing cell viability, altering oxidative stress levels, and inducing cell death. Up to this point, little research has been done to test the efficacy of metal cyanoximates and their use in cancer therapy. In studies by Ratcliff, Kudok-Jaworska et al. and Dannen, Cornelison et al., the efficacy of several different metal cyanoximates, Pt (MCO)₂, Pd (MCO)₂, Pt (DECO)₂ and Cisplatin on reducing cell viability was investigated (2012 and 2020, respectively). The Dannen study tested this with a trypan blue assay, which determined that both Cisplatin and Pt (DECO)₂ were able to reduce cell viability *in vitro* (2020, submitted). In the study, viability of cervical cancer cells (HeLa) was reduced by nearly 60% by Pt (DECO)₂ and reduced by 55% in the positive control of Cisplatin-treated cells (Appendix A). Additionally, WiDr, a colon cancer cell line, was treated with 1.0 mM Cisplatin or Pt (DECO)₂, and 95% of colon cancer cells were killed by Pt (DECO)₂ but only 12% by Cisplatin (Appendix A). In the Ratcliff and Dannen papers, trypan blue assay was employed to differentiate between live and dead cells as the dye only penetrates dead cells with damage to their plasma membrane (Piccinini, Tesei et al., 2017). The main disadvantages to this technique include subjectivity judgement of the user to differentiate between stained or unstained cells, inconsistency among different users, the amount

of time and labor involved with measuring samples, and the error involved with only measuring single samples at a time (Piccinini, Tesei et al., 2017). Whereas with XTT, the tetrazolium salt is only getting reduced by metabolically active or viable cells and quantified automatically with a spectrophotometer. Even so, our study utilizing XTT showed similar results to the Dannen paper in that 1.0 mM Pt (DECO)₂ reduced viability more effectively (by nearly 59% in thyroid cancer cells), than Cisplatin did (only 45% of cells were killed by 1.0 mM Cisplatin treatment). Based on results presented in the Eddings and Dannen papers, Pt(DECO)₂ ranks in third place when compared to other platinum or palladium-based cyanoximates when treating cervical or colon cancer cells *in vitro* (Table 1). However, in all three cell lines (HeLa, WiDr, and Thyroid), Pt(DECO)₂ is shown to be more effective than Cisplatin at the same concentration (Dannen, Cornelison et al., 2020). It is thought that the oxime ligand, DECO, alongside the platinum molecule has a more efficient structure to confer lethality than Cisplatin's square planer model (Ratcliff, Kudok-Jaworska et al., 2012; Dannen, Cornelison et al., 2020), or that the combination of platinum with the oxime molecules in conjunction is more effective than platinum bound to chloride or ammonium as seen in Cisplatin.

Reduction of ROS

I found that the platinum-based nanoparticles actually reduced the level of superoxide formed upon treatment, similarly to what was seen in the Yusof, Akmal et al. paper that described the use of platinum nanoparticles as an antioxidant in treating lung disease (2015). They assessed the use of platinum-based nanoparticles as an alternative drug carrier due to their ability to produce less of an ROS-mediated effect to the system and suggested that platinum may reverse the oxidative imbalance by affecting the epithelial sodium channel, or ENaC. ENaC

works by downregulating the Protein Kinase C (PKC) pathway in the lungs and showed great promise in the treatment of lung disease (Yusof, Akmal et al., 2015). In a paper by Shibuya, Ozawa et al., it was seen that platinum nanoparticles share similar activity to that of catalase and superoxide dismutase, which are key endogenous antioxidant enzymes found in human systems (2014). Moreover, in a stroke mice model done by Takamiya, Miyamoto et al. (2011) there was a significant reduction in the production of superoxide upon platinum treatment. This is in agreement with reduced levels of superoxide observed in my study, and offers insight as to the mechanism behind superoxide reduction. In contrast, non-treated cancer cells display increased oxidative stress that can lead to damage in the DNA, proteins, and lipids (Li, Jia et al., 2016).

Induced Apoptosis

Programmed cell death, or apoptosis is the body's natural mechanism for killing cells (Goldar, Khaniani et al., 2015). Apoptosis is carried out through pathways that utilize caspases to signal a cell for death (Pfeffer and Singh, 2018). In cancer pathology, these apoptotic pathways are inhibited through overexpression of anti-apoptotic proteins and under-expression of proapoptotic proteins (Pfeffer and Singh, 2018). Due to these changes, many cancers can develop a resistance to anticancer chemotherapies. Therefore, there is an unmet need to develop new anticancer drugs that can activate these apoptotic pathways. In my study, it was seen that Cisplatin and Pt (DECO)₂ both caused significant amounts of apoptosis. Moreover, it is suggested that Pt (DECO)₂ was more efficient in inducing cell death as seen by the increase in apoptosis above (Figure 6F and 7F). Additionally, it can be concluded that neither Cisplatin or Pt (DECO)₂ had much specificity in the killing of rapidly dividing cells, regardless if they were cancerous or not, as seen in Figure 7. In the Dannen, Cornelison et al. paper, healthy C₅₇BL/6

mice were injected intraperitoneally with 1.0 mM Cisplatin and Pt (DECO)₂ to assess cytotoxicity (2020). Their results suggest a less severe side effect profile compared to Cisplatin; however, enhanced hepatotoxicity was not addressed, even with it being a hallmark of many chemotherapies (Gong, Qian et al., 2015).

Limitations to *in vitro* Studies

Through the years, *in vitro* studies have helped expand our knowledge and understanding of the mechanisms of toxicity that can happen *in vivo*. However, it is important to understand that even with the great deal of benefits these studies provide, there are still several limitations to their overall reflection of what happens in an actual human system. For example, the human body has a complex network of organs and organ systems that behave differently and compensate to different stimuli and stress situations that cannot be replicated *in vitro* with a single cell type (Aslantürk, 2017). Even with simple assays that test metabolism of different chemicals, such as with an XTT assay, drug metabolism will vary in an actual human system (Aslantürk, 2017). Furthermore, even primary cultivated cells tend to differ greatly from their corresponding cell type in an actual organism, so it's hard to predict if outcomes are going to be fully replicable (Aslantürk, 2017) outside of exact physiological conditions. For example, Godoy, Hengstler et al. revealed that primary hepatocytes when isolated from their normal microenvironment display hundreds of genes that are differentially expressed and either up or downregulated differently than what is seen in animal models (2009).

CONCLUSION

Incidence of thyroid cancer is on the rise with over fifty-two thousand new cancer diagnoses in the US each year. Although much is still unknown regarding the exact cause of thyroid cancer, researchers hope to find better treatments for patients battling thyroid cancers. Results from my study demonstrated that CTX-SAP decreased cell viability of ML-1 cells that express MMP-2, which points towards MMP-2 as a potential target for RAI-resistant thyroid cancer. However, further studies are needed to develop safe and effective treatments against aggressive thyroid cancer using CTX-SAP. Since the discovery of the first platinum-based anti-cancer drug, Cisplatin, a great deal of research has been conducted in elucidating better alternative platinum or metal-based anti-cancer drugs. Due to the adverse side effects associated with treatment, researchers are looking to develop and validate selective metal-based anti-cancer drugs for cancer therapy. Presently, *in vivo* studies utilizing healthy mice show great promise in reducing these chemotherapy-induced side effects (Dannen, Cornelison et al., 2020). However, challenges still remain and more research needs to be done in an animal cancer model to determine how intraperitoneal administration affects the entire organism. My study demonstrated that Pt (DECO)₂ has potential for use as an anti-cancer drug. Further studies are needed to develop safe and effective treatment against aggressive thyroid cancer using this cyanoximate as an alternative to existing platinum-based anti-cancer drugs. Current research shows promise of Pt (DECO)₂ being more effective than Cisplatin at higher doses. However, more research needs to be done to test efficacy of both at lower doses and their ability to be used in conjunction with each other as a combination therapy. Moreover, if the ability to conjugate Pt (DECO)₂ to an MMP-2 receptor agonist like Chlorotoxin would allow for the successful targeting of this drug to just cancerous cells and not healthy cells too.

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Table 1: American Cancer Society Information & Statistics on Thyroid Cancer

Type of Thyroid Cancer	Signs & Symptoms	Description	Occurrence
Papillary (PTC)	Slowly develop in one lobe of thyroid gland & spread to LN. (DTC)	Most common, considered differentiated	8 out of 10 of all thyroid cancers
Follicular (FTC)	Rarely spread to LN, but do spread to other body parts. (DTC)	Second most common. More common in iodine-insufficient diets	1 out of 10 of all thyroid cancers
Medullary (MTC)	Can spread to LN, lungs, or liver before nodule present. Alters blood-calcium levels	Develop in the C cells of thyroid gland. Can be familial. Linked to increased risk of having other tumors.	4% of all thyroid cancer cases
Anaplastic (ATC)	Cancer cells do not resemble normal thyroid cells (UTC)	Most rare, very aggressive and difficult to treat, can metastasize quickly to other parts of the body.	2% of all thyroid cancers

Source: American Cancer Society: What is Thyroid Cancer?

<https://www.cancer.org/cancer/thyroid-cancer/about/what-is-thyroid-cancer.html>

DTC – Differentiated Thyroid Cancer

UTC – Undifferentiated Thyroid Cancer

Table 2: National Cancer Institute Treatment Recommendations for Thyroid Cancer

Type of Thyroid Cancer	Disease Status	Treatment Options
Papillary & Follicular	Localized/regional	Surgery (total thyroidectomy or lobectomy), RAI, TSH, EBRT
Papillary and follicular	Metastatic	Iodine-Sensitive: RAI or Thyroid-suppression therapy Iodine-resistant: Thyroid-suppression, targeted therapy, Surgery, or EBRT
Recurrent papillary and follicular	-	Surgery (W/ or W/o Post-Op RAI), targeted therapy, EBRT, or Chemotherapy
Medullary	Localized disease	Total thyroidectomy, EBRT
Medullary	Metastatic	Targeted therapy, Palliative Chemotherapy
Anaplastic	-	Surgery, EBRT, Systemic Therapy

Source: National Cancer Institute. Thyroid cancer treatment (PDQ).

www.cancer.gov/cancertopics/pdq/treatment/thyroid/HealthProfessional.

RAI – Radioactive Iodine

TSH – Thyroid Stimulating Hormone

EBRT – External Beam Radiation

Table 3: Solubility of All Three Platinum Complexes When First Solubilized in 100 μ L DMSO and Diluted with DMEM

Toxins	1% DMSO		2 % DMSO *		4 % DMSO	
	0 hr.	24 hr.	0 hr.	24 hr.	0 hr.	24 hr.
Pt (DECO) ₂	Soluble	Soluble	Soluble	Soluble	Soluble	Soluble
Pd (DECO) ₂	Insoluble	Insoluble	Insoluble	Insoluble	Soluble	Soluble
Cisplatin	Soluble	Soluble	Soluble	Soluble	Soluble	Soluble

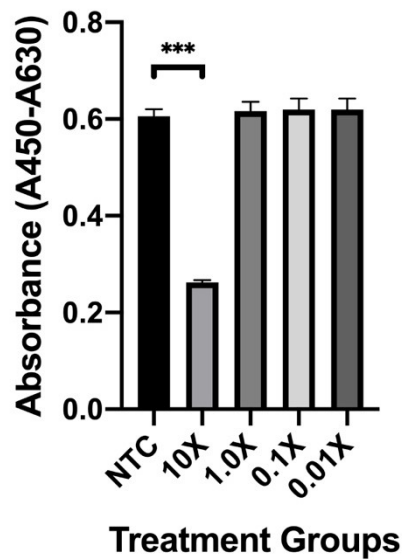


Figure 1: Measurement of cellular viability of thyroid cancer cells via reduction of XTT salt. Data represents absorbance values/relative cell viability (A450-A630) at differing concentrations (in percentages) of DMSO. A “***” represents $p \leq 0.0001$. Results display mean and SD of triplicate sets.

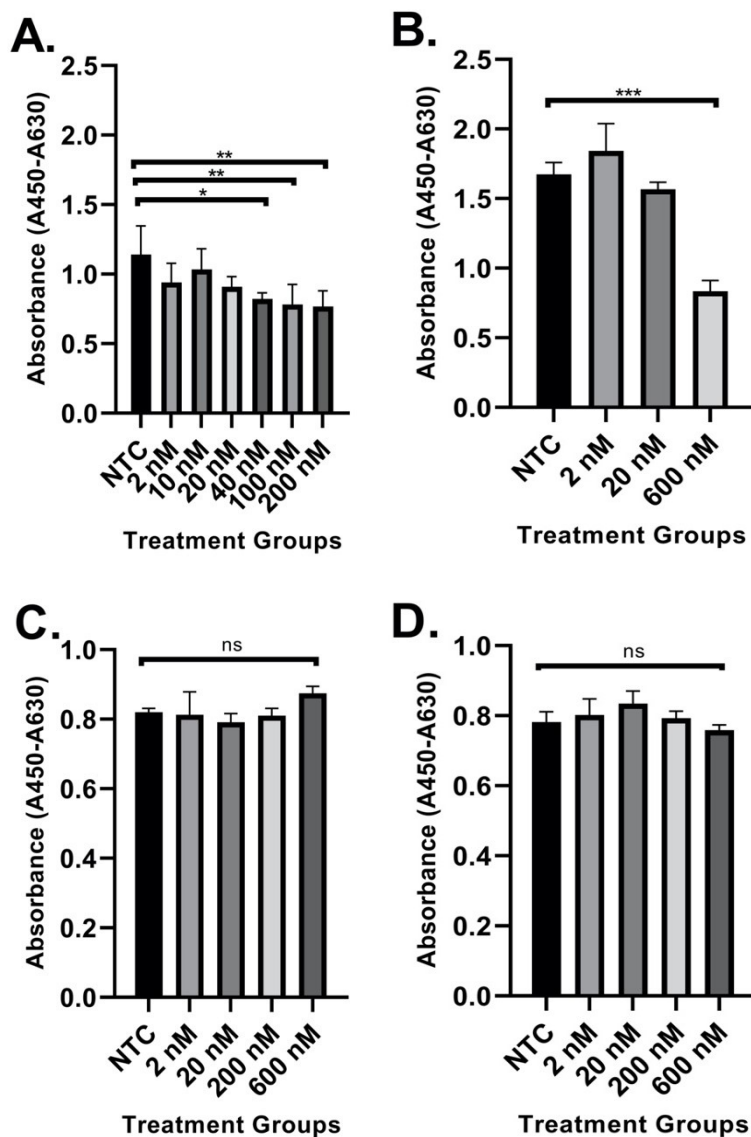


Figure 2: Measurement of cellular viability via reduction of XTT. Data represents absorbance values/ relative cell viability (A450-A630) at differing concentrations of (A) 0 nM – 200 nM CTX-SAP, (B) 0 nM – 600 nM CTX-SAP, (C), 0 nM – 600 nM CTX, and (D) 0 nM – 600 nM SAP treatment compared to a non-treated control (NTC). A “*” represents $P \leq 0.05$, “**” represents $P \leq 0.01$, and “***” represents $p \leq 0.0001$. Results display mean and SD of triplicate sets.

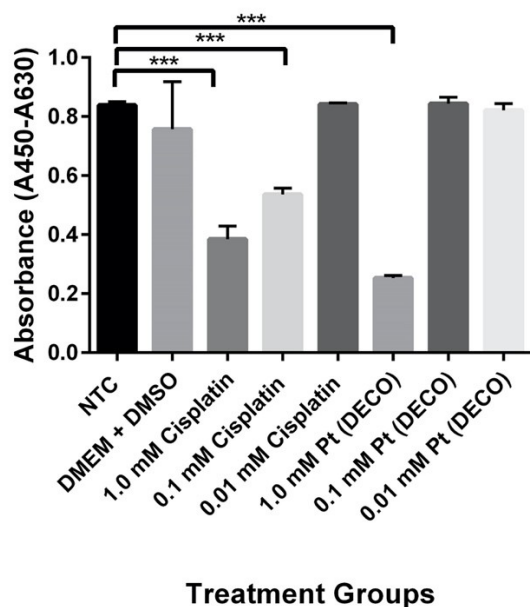


Figure 3: Measurement of thyroid cancer cell viability via reduction of XTT salt in ML-1 thyroid cancer cells. Data represents absorbance values/ relative cell viability (A450-A630) at differing concentrations of Cisplatin and Pt (DECO)₂ treatment compared to a non-treated control (NTC). A “****” represents $p \leq 0.0001$. Results display mean and SD of triplicate sets.

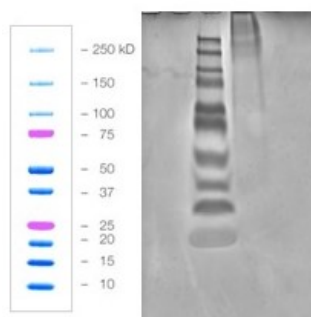


Figure 4: Native Gel compared with BIO-RAD protein ladder.

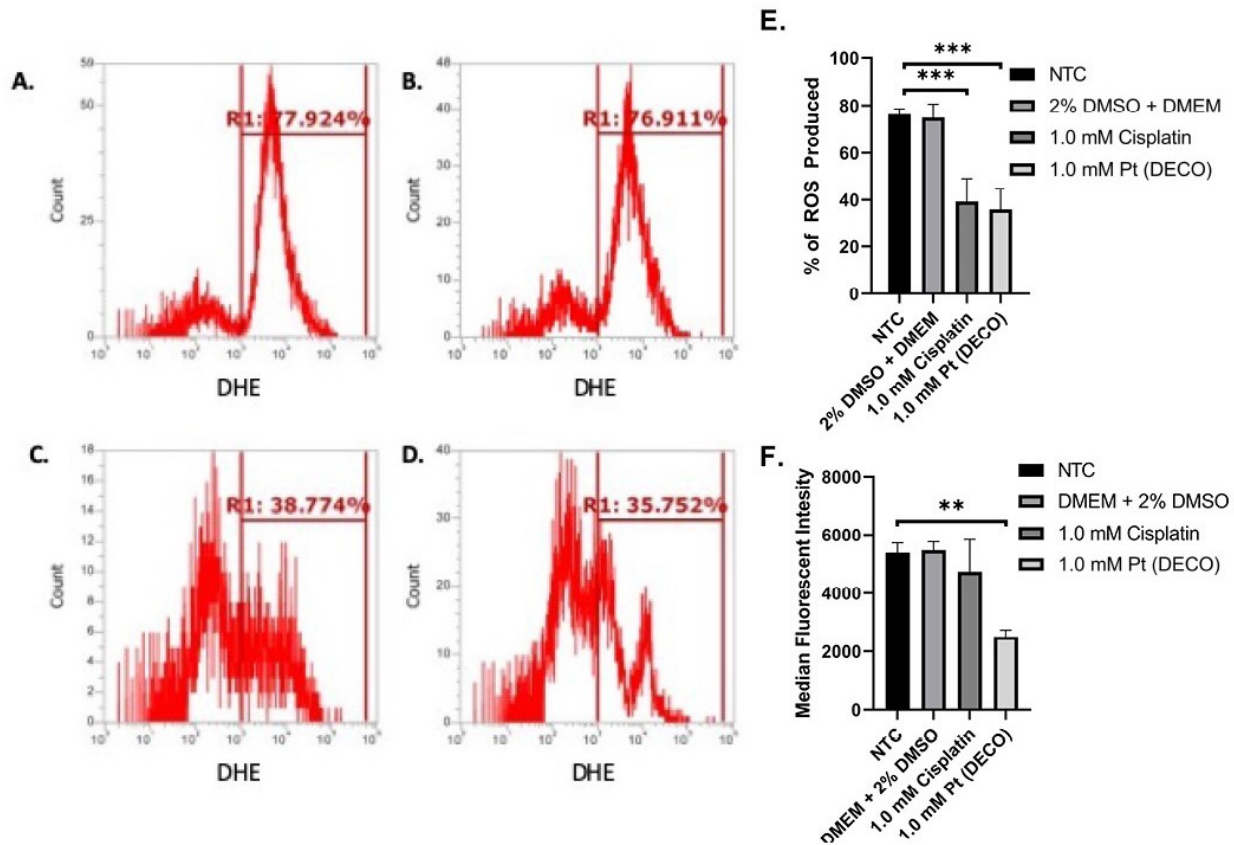


Figure 5: Quantification of superoxide production in ML-1 thyroid cancer cells using dihydroethidium (DHE) as standard and analysis at 518/606 nm. “A” shows the fluorescent intensity of DHE on cells not treated with either drug or DMSO. “B” displays the fluorescent intensity of DHE on cell treated with 2% DMSO. “C” shows fluorescent intensity of DHE on cells treated with 1.0 mM Cisplatin. “D” displays fluorescent intensity of DHE on cells treated with 1.0 mM Pt (DECO)₂. “E” shows the total percent of ROS produced by each treatment group done in triplicate. “F” shows the mean fluorescent intensity of each group. “***” represents $P \leq 0.01$, and “****” represents $p \leq 0.0001$. Results display mean and SD of triplicate sets.

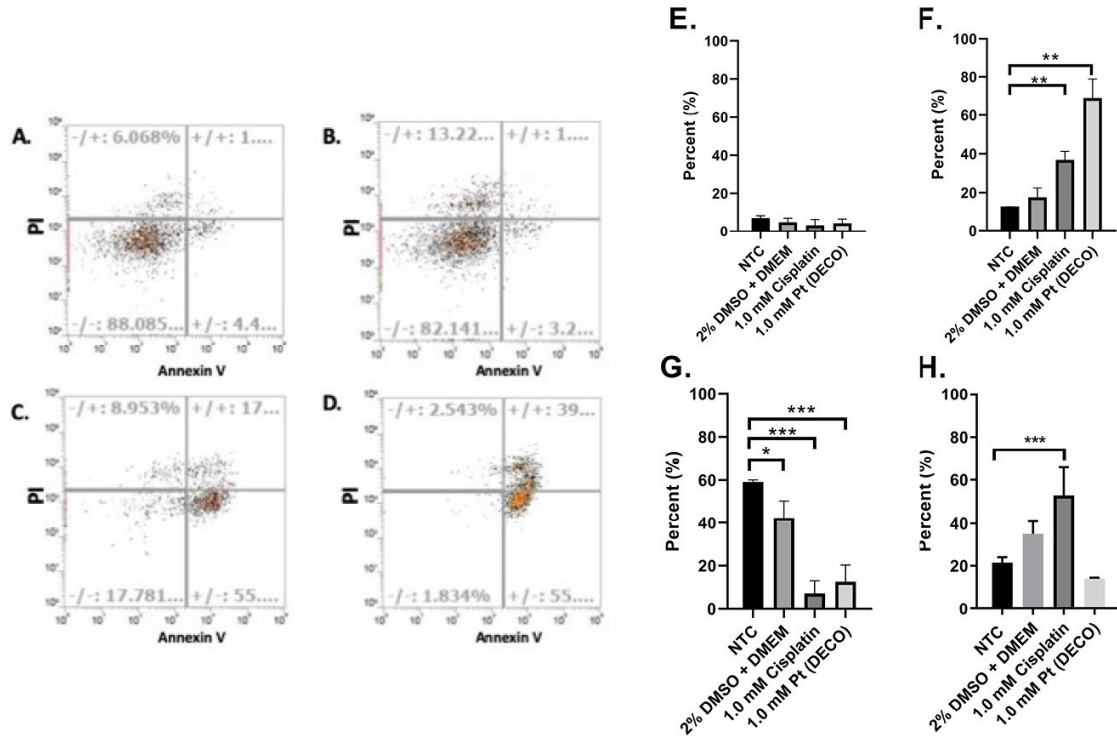


Figure 6: Percent of cancer cells presenting at a particular stage of programmed cell death under Annexin V-APC (650nm) and Propidium Iodide (620 nm) after 24 hours of treatment. A-D represent fluorescent intensity of the dyes on each treatment group. “A & E” displays the percent of necrotic cells present, “B & F” shows cells presenting signs of late apoptosis, “C & G” displays the percentage of viable cells, and “D & H” shows cells displaying early apoptosis. A “*” represents $P \leq 0.05$, “**” represents $P \leq 0.01$, and “***” represents $p \leq 0.0001$. Results display mean and SD of triplicate sets.

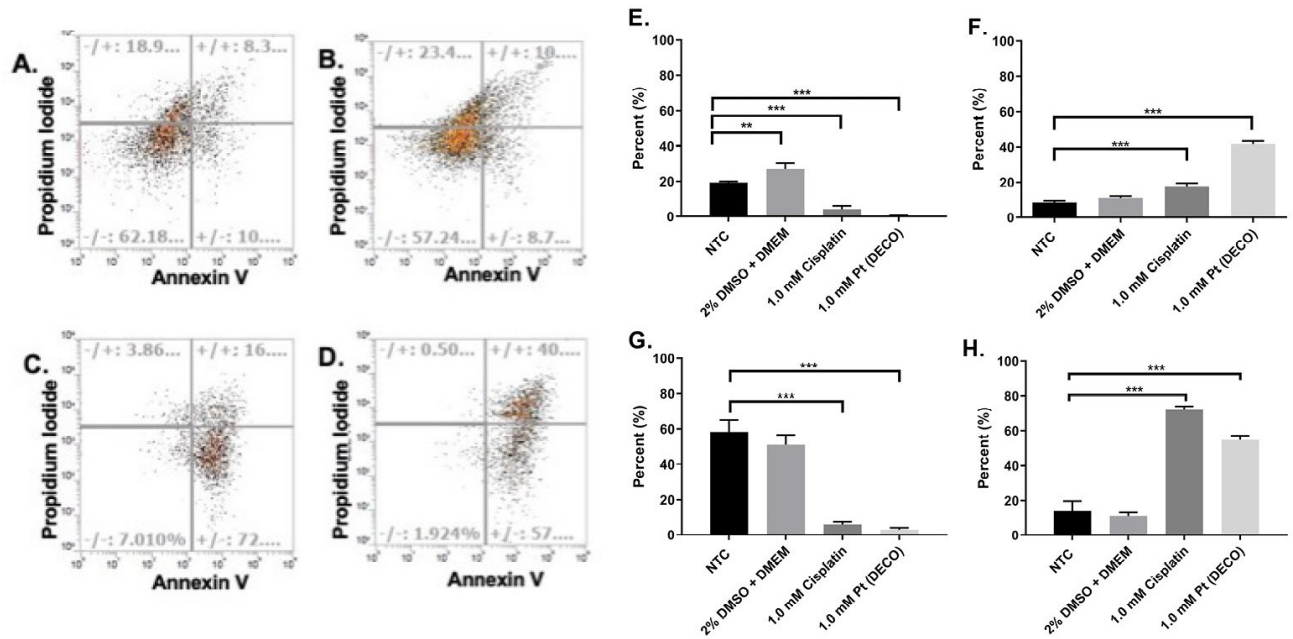


Figure 7: Percent of Fibroblast cells presenting at a particular stage of programmed cell death under Annexin V-APC (650nm) and Propidium Iodide (620 nm) after 24 hours of treatment. “A” displays the percent of necrotic cells present, “B” shows cells presenting signs of late apoptosis, “C” displays the percentage of viable cells, and “D” shows cells displaying early apoptosis. “**” represents $P \leq 0.01$, and “***” represents $p \leq 0.0001$. Results display mean and SD of triplicate sets.

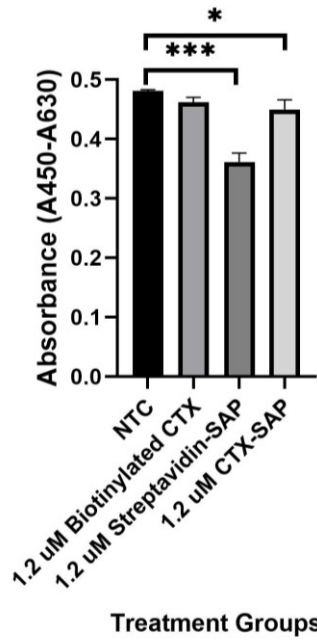


Figure 8: Measurement of cellular viability via reduction of XTT. Data represents absorbance values/relative cell viability of thyroid cancer cells (A450-A630) at differing concentrations of internally-made CTX-SAP at 0 – 1.2 uM concentrations compared to a non-treated control (NTC). A “*” represents $P \leq 0.05$, and “***” represents $p \leq 0.0001$. Results display mean and SD.

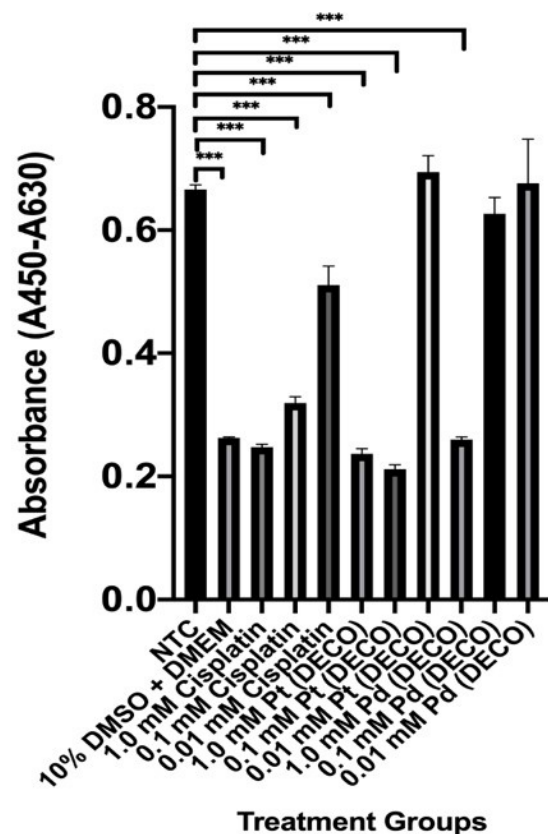


Figure 9: Measurement of cellular viability of thyroid cancer cells via reduction of XTT salt. Data represents absorbance values/ relative cell viability (A450-A630) at differing concentrations of Cisplatin and Pt (DECO)₂ solubilized in 10% DMSO compared to a non-treated control (NTC). A “***” represents $p \leq 0.0001$. Results display mean and SD.

APPENDICES

Appendix A. Additional Resources

Appendix A: Comparison of Cyanoximates effects in vitro.

Cyanoximate (at 1.0 mM)	Viability Reduced by (%):		Rank	
	HeLa	WiDr	HeLa	WiDr
Cisplatin	55/56	12	4	6
Pt(MCO) ₂	42	73	6	5
Pd(MCO) ₂	45	81	5	4
Pt(DECO) ₂	60	95	3	3
Pd(DECO) ₂	94	98	2	1
Pt(PyrCO) ₂	95.2	96.4	1	2
Pd(PyrCO) ₂	-	-	Mixed results (N/A)	

Data from Dannen, Cornelison et al., paper, submitted in January of 2020.
Each drug was solubilized in 1% DMSO.

Appendix B. Online Resources

American Cancer Society. 2019 Treatment of Thyroid Cancer, by Type and Stage. Available at: [www. https://www.cancer.org/cancer/thyroid-cancer/treating/by-stage.html](https://www.cancer.org/cancer/thyroid-cancer/treating/by-stage.html) (accessed June 14, 2019).

National Cancer Institute n.d. Cancer Stat Facts: Thyroid Cancer. Available at: [www.https://seer.cancer.gov/statfacts/html/thyro.html](https://seer.cancer.gov/statfacts/html/thyro.html) (accessed June 13th 2018).

National Institutes of Health (US) 2007 Understanding Cancer. Available at: [www.https://www.ncbi.nlm.nih.gov/books/NBK20362/](https://www.ncbi.nlm.nih.gov/books/NBK20362/) (accessed August 8, 2019).

XTT Cell Proliferation Assay Kit: Instruction Manual. 2011. Available at: <https://www.atcc.org/~media/Attachments/5/6/3/7/16747.ashx> (accessed June 13th 2018).