Novel Cyanoximates as an Alternative in Cancer Chemotherapy

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NOVEL CYANOXIMATES AS AN ALTERNATIVE IN CANCER CHEMOTHERAPY

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

Kafayat Aderonke Yusuf

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NOVEL CYANOXIMATES AS AN ALTERNATIVE IN CANCER CHEMOTHERAPY

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ABSTRACT

Chemotherapy is one of the most effective treatment plans for several cancer types. The recurrent side effects derived from chemotherapy agents have warranted the search for novel chemical compounds with better efficacy and minimal side effects. In line with this idea, I investigated effects of a group of newly synthesized metal based chemical compounds called cyanoximates on HeLa human cancer cells. Cyanoximates used were Pt(DECO)$_2$, Pt(MCO)$_2$, and Pd(DECO)$_2$ along with the chemotherapy drug cisplatin as a positive control. I found that the metal cyanoximates reduced cell viability via apoptosis, and that Pt(DECO)$_2$ was most effective among these new cyanomixates. In an attempt to understand the potential mechanism of action of Pt(DECO)$_2$, I performed RNAseq analysis with HeLa cells treated with 0.5 mM Pt(DECO)$_2$. Hundreds of genes in Pt(DECO)$_2$-treated cells were differentially expressed with several upregulated genes known to be involved in cell cycle regulation and apoptosis. The analysis also revealed that cancer growth promoting genes alongside drug transporter genes are downregulated in the presence of Pt(DECO)$_2$. Taken together, these results provide evidence that Pt(DECO)$_2$ can be an alternative agent for cisplatin-based chemotherapy, and further, the transcriptomic analysis offers new insights into the mechanism of action of Pt(DECO)$_2$ against cancer cells.

KEYWORDS: cancer, chemotherapy, cisplatin, cyanoximates, apoptosis, p53, HeLa cells
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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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I dedicate this thesis to Almighty Allah, the beneficent, the merciful.
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INTRODUCTION

Cancer can be described as a disease that involves a group of abnormal cells growing out of control, disregarding the normal patterns of cell division (Hejmadi, 2013). Ideally, normal dividing cells are constantly regulated by cues that dictate whether the cells should divide, differentiate into another cell, or undergo apoptosis (Hejmadi, 2013). Over time, cancer cells become resistant to these signals, becoming autonomous to cell growth and division prompts, and consequently leading to uncontrolled cell division, growth, and proliferation (Ferlay et al., 2015; Hejmadi, 2013). Over 10 million new cases of cancer are reported each year and cancer-related deaths are projected to increase in the near future with an estimation by the World Health Organization of approximately 13 million cancer-related deaths by the year 2030 (Ferlay et al., 2015). Generally, cancer cells are believed to develop from normal cells due to damage of DNA. Typically, when DNA becomes damaged, the body is able to repair it. Unfortunately in cancer cells, damaged DNA isn’t repaired and occasionally can be inherited (Sudhakar, 2009).

History and Causes of Cancer

Cancer has been recognized for several hundred years, with its earliest description in a book titled “Cautions against the immoderate snuff” written by physician John Hill (Hill, 1761). The book was published in 1761 as a result of scientific findings that tobacco snuff caused “polypus”, which was described as a small vascular growth on the epithelium of a mucous membrane (Hill, 1761; Basu, 2018). No significant studies had been made till over two decades later when Hill's findings paved the way for new studies on cancer epidemiology where it was noted that tobacco smoking caused lung cancer (Basu, 2018). The first occupational link to
cancer was reportedly established in 1775 when Sir Percivall Pott of Saint Bartholomew’s
Hospital in London wrote an essay that linked soot leads to high incidence of scrotal cancer in
young men that worked as chimney sweeps, which he then referred to them as the chimney-
sweepers’ cancer (Taxell and Santonen, 2017; Vincent et al., 2008). No significant studies on
carcinogens were published till the early 20th century when two Japanese investigators
developed the first animal assay for carcinogens. They repeatedly applied coal tar on the skin of
rabbit ears, which resulted in the development of tumors after a few weeks. Subsequently, rats
and mice were found to be more responsive to the carcinogens (Basu, 2018). Over the years,
many compounds and mixtures have been recognized as potential human carcinogens. Numerous
findings have illustrated that asbestos, chromium, nickel, and arsenic compounds, vinyl chloride,
aflatoxins and most notably tobacco smoke are potential carcinogens (Hayes, 1997; Nicholson,
2001; Purohit and Basu, 2000). It has also been reported that there is a high rate of cancer
occurrence in patients with viral infections from Hepatitis B and C virus, human papillomavirus
(HPV), as well as human immunodeficiency virus (HIV) that have contributed to cases of
cervical cancer, liver cancer, lymphomas, carcinomas, and nasopharyngeal cancers (Hejmadi,
2013; Sudhakar, 2009; Wogan, 1999). Prolonged sun exposure is another factor causing skin
damage, due to increased exposure to ultraviolet rays from the sun; the fast rise in tanning salons
have also fostered the increased rate of skin cancers (melanomas) (Robock and Toon, 2010;
Soehnge et al., 1997).

**Cervical Cancer**

Cervical cancer has been described as one of the most common gynecological tumors that
affect adult women (Waldmann, et al., 2013). According to recent statistics, about 569,847
women are pronounced of having cervical cancer annually, leading to over 311,365 deaths (Bray
et al, 2018). It is believed that infection with human papillomavirus (HPV) is a necessity for the development of cervical cancer (Walboomers et al., 1999); this is attributed to the detection of HPV DNA in 90% of cervical cancers (Waldmann, et al., 2013). Even with the advent of HPV vaccines and numerous improvements in cervical screening (Goodman, 2015), cervical cancer still ranks second in terms of high mortality among gynecological tumors in developing countries (Shasrabuddhe et al, 2012). The 5 year overall survival of metastatic cervical cancer estimates at only 15% with limited treatment options been the ultimate cause (Guitarte et al, 2014). In recurrent/metastatic cervical cancer, majority of the affected patients are subjected to palliative chemotherapy (Boussios et al, 2016) in which platinum-based chemotherapies were the topmost choice (Monk et al. 2009).

**Cancer Prevention and Treatment**

Findings have suggested that the risk of getting cancer can be minimized by lifestyle changes and modifications (Hejmadi, 2013; Stein and Colditz, 2004). Primary cancer prevention includes quitting smoking and minimized alcohol consumption (Stein and Colditz, 2004). Likewise, diet modification through the incorporation of fruits and vegetables as well as a high fiber diet can help reduce the incidence of stomach and colon cancer (Anand et al., 2008; Hejmadi, 2013). Currently used treatment for cancer includes surgery, radiation, chemotherapy, and sometimes hormonal blocking (Senapati et al., 2018). Surgery becomes an ineffective process in cancer treatment when the tumor has spread to different parts of the body. Radiation therapy, although effective, is also limiting because healthy cells are harmed in the process, and therefore, newer radiation machines that concentrate the energy to only cancerous cells are being considered (Chandarana et al., 2018). Chemotherapy involves the use of anticancer medications. In particular, this therapy is often prescribed when cancerous cells have spread widely in the
body, but most times a combination of such medications works better than a single medication (Bayat et al., 2017; Vincent et al., 2008). Cancer chemotherapy has also been reported to achieve partial or complete response where either half or all of detectable cancerous cells are eliminated. The set back of chemotherapy use is the development of drug resistance by cancerous cells over time (Housman et al., 2014)

Cancer Chemotherapy

A famous German Chemist Paul Ehrlich coined the word “chemotherapy” and defined it as the use of chemicals to treat disease (Vincent et al., 2008). Chemotherapy works by killing rapidly dividing cells, halting their growth and spread or slowing down their development. Chemotherapy can, however, affect healthy cells, leading to unwanted side effects (Yimit et al., 2019). The use of various metal based compounds in treatment of several diseases is dated to ancient times due to their therapeutic properties (Frezza et al., 2010). Metals are part of numerous biological activities, such as reaction catalysts, and structural functions to the exchange of electrons in cells. They are often situated in the enzyme catalytic domains and are profoundly employed in cellular activities. These metals are often needed in just trace amount to facilitate catalytic events in the cell (Bruijninx and Sadler, 2008). In spite of the prevalence of metal based compounds in the treatment of diseases since ancient times, lack of appropriate dosage was a major drawback associated with its use. Medical experts at that time had little or no knowledge about dose-related biological response, but the emergence of cell biology, molecular biology, and biochemistry paved the way for the design of metal based compounds to target specific molecules in the cell (Ji et al., 2009).
The advent of the platinum based compound cisplatin in 1960 by Barnett Rosenberg (Jungwirth et al., 2011; Pranczk et al., 2014) was a major advancement in the use of metal based compounds in the treatment of cancer cells. This major breakthrough was considered the foundation for development of modern day metal based anticancer drugs (Jungwirth et al., 2011). Distinctive features of metals such as reactivity towards organic substrate, variable coordination modes, and redox activity has enhanced their use in cancer therapy (Frezza et al., 2010). These unique characteristics were the attractive site of metals in the model of metal complexes that binds to the biomolecular target with a resultant disruption in the mechanism of cell proliferation ultimately leading to death of the cells. The recent groups of metal based synthesized compounds are designed to achieve higher levels of cytotoxicity and lesser side effects that could not be achieved with the compounds already in use (Ndagi et al., 2017). Generally, platinum compounds, particularly cisplatin, are the most prevalent of the metal based compounds used in cancer chemotherapy (Dasari and Tchounwou, 2014). Platinum complexes act as an adjuvant in cancer treatment to facilitate apoptosis or programmed cell death (Florea and Busselberg, 2011). These metal complexes are mostly recommended for the treatment of ovarian, testicular, breast, ovarian, prostate, and lung cancers (Basu and Krishnamurthy, 2010; Jung and Lippard, 2007).

For over three decades now, platinum based anticancer drugs, including cisplatin and its analogues, have dominated the treatment of cancers by chemical events (Abu-Surrah et al., 2008; Dasari and Tchounwou, 2014, Kostova, 2006). The discovery of cisplatin (cis-diaminedichloro-platinum(II)), decades ago marked the beginning of a tremendous development in the production of platinum based anticancer drugs (Orvig and Abrams, 1999). Cisplatin is a very efficient anticancer drug, with several positive results against testicular, ovarian, bladder tumors, as well as head and neck malignancies (Ho et al., 2016). Research has indicated that cisplatin is usually
administered intravenously rather than orally because of its low solubility in water (Kostova, 2006; Pranczk et al., 2014). The mechanism of cisplatin action is by passive diffusion (Jamieson and Lippard, 1999), and the cytotoxicity of cisplatin is believed to arise from its binding to DNA and the formation of covalent cross-links (Jamieson and Lippard, 1999). Binding of cisplatin to DNA then causes alteration of helical structure and results in halting of DNA replication and transcription (Lee et al., 2002). The disrupted DNA structure can be used as a recognition or binding site for cellular proteins such as repair enzymes, transcription factors, histones and high-mobility group (HMG) proteins (Kartalou and Essigmann, 2001). The process of binding of the HMG proteins to cisplatin-DNA lesions has been suggested to facilitate the antitumor activity of the drugs (Kane and Lippard, 1996). The anticancer property of cisplatin is also influenced by the efficiency of cisplatin-DNA adduct removal by the cellular repair machinery, with nucleotide excision repair being a major pathway (Kostova, 2006). Another commonly used platinum based drug is, carboplatin (diamine[1,1-cyclobutanedicarboxylato(2-)]-O,O´-platinum (II)) (Sakurai et al., 2002). The basic pharmacokinetic differences between cisplatin and carboplatin depend primarily on the slower rate of conversion of the latter to reactive species. Regarding the interaction of carboplatin with DNA, results from studies provide evidence that the reaction is initiated via ring-opening in carboplatin and subsequent binding with DNA constituents. The side effects of using carboplatin is also described as minimal when compared with cisplatin, and the drug is said to be better tolerated by the body and can be used at higher prescription doses than cisplatin (Kapdi and Fairlamb, 2014). Oxaliplatin is another platinum based anticancer drug that has been approved for use against cancer cells that do not respond to cisplatin. Its mechanism of action is not completely understood but it is believed to interact with DNA in a
similar way to cisplatin and carboplatin. Oxaliplatin can be employed for the use with other medications against the treatment of advanced colorectal cancer (Kapdi and Fairlamb, 2014).

Platinum and palladium are believed to have similar physical and chemical properties. The significant similarity between the coordination chemistry of palladium (II) and platinum (II) compounds has triggered the studies of Pd (II) complexes as antitumor drugs (Kapdi and Fairlamb, 2014). Likewise, hydrolysis of palladium is about $10^5$ times faster than its platinum counterpart, which enables it to dissociate instantly in solution and ultimately is unable to reach its pharmacological targets (Abu-Surrah et al., 2008). Research has indicated that one of the methods to improve the biological activity of Pd (II) complexes is its combination with selected ligands that will significantly decrease the rate of hydrolysis at the metal site (Ratcliff et al., 2012b). Several studies have focused on the preparation of Pd (II) complexes bearing bidentate ligands as a way to stabilize these compounds and to prevent any possible cis-trans isomerism (Mansuri-Torshizi et al., 1992).

A currently studied group of ligands called cyanoximes, is the common name for a family of compounds with the general formula NC-(C≡N-OH)-R, where R is an electron-withdrawing group (Gerasimchuk et al., 2008). Cyanoximes have been described as outstanding ligands with unique biological properties such as growth regulation, antimicrobial, and detoxification activities (Gerasimchuk et al., 2008; Ratcliff et al., 2012a). In line with the idea of further exploration of new, non-traditional platinum complexes, cytotoxic bivalent Pd and Pt complexes with deprotonated MCO and PiPCO ligands were shown to possess in vitro activity comparable to cisplatin (Ratcliff et al., 2012a). These square-planar Pd and Pt complexes represent the first group of active, novel oxime-based bis-chelates. However, prior to the above studies, no high-yield preparations of Pd, Pt-cyanoximates were developed, no direct structural information about
these complexes was available, and their electronic structure was not fully understood (Ratcliff et al., 2012b). Studies have revealed that cyanoximes are excellent ligands for a strong binding of a variety of metal ions in formed complexes, and they are considered much better ligands than traditional aldoximes and ketoximes (Marcano et al., 2015). The anti-proliferative activity and cytotoxicity of cyanoximes and their metal complexes have not been adequately studied. The first bivalent palladium and platinum cyanoximate was synthesized, characterized and described by Dr. Nikolay Gerasimchuk of the chemistry department in conjunction with Dr. Paul Durham of the biology department Missouri State University (Eddings et al., 2004). This paper highlighted the *in vitro* biological activity of bivalent Pd and Pt cyanoxime complexes with ligand derivatives of substituted cyan-acetamides abbreviated as HPiPCO and HMCO (Ratcliff et al., 2012b). Recently, Dr. Gerasimchuk also synthesized a new group of cyanoximates; Platinum and Palladium DECO, (DECO:2-cyano-2-oxiamino-N,N’-diethylaminoacetamide), Bis (2-cyano-2-oxiamino-N,N’-diethylaminoacetamide) platinum (Pt(DECO)₂) and Bis (2-cyano-2-oxiamino-N,N’-diethylaminoacetamide) palladium (Pd(DECO)₂) (Klaus et al., 2015). There is still limited information about the potentials of the newly synthesized cyanoximates.

**Reactive Oxygen Species and Chemotherapy**

Reactive oxygen species (ROS) has been described as unstable, reactive, partially reduced oxygen derivatives that are generated as an end product of metabolism. They include hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), singlet oxygen (¹O₂), hydroxyl radical (·OH) and sometimes peroxynitrites (ONOO⁻) (Chio and Tuveson, 2017) that acts as a borderline between reactive oxygen and reactive nitrogen species. They are believed to act as secondary messengers in cell signaling and are essential for various biological processes in healthy and
diseased cells (Chio and Tuveson, 2017). Altering of ROS levels is a mechanism shared by most chemotherapeutics due to their effect in promoting programmed cell death (Conklin, 2004). In cancer cells, ROS levels are increased due to both environmental and internal mechanisms. The overall balance of ROS and the combined positive and negative effects of ROS all contribute to the final impact on cancer biology (Yang et al., 2018). Most chemotherapeutics generate ROS in cancer cells, and findings from several studies indicate that chemotherapeutic amplification of ROS levels pushes the already increased cancer cells over a threshold to cause cell death and is one of the proposed mechanisms by which multiple chemotherapies foster tumor regression (Pelicano et al., 2004; Yang et al., 2018). Anthracyclines, such as doxorubicin, daunorubicin and epirubicin, are believed to produce the highest levels of cellular ROS (Li et al., 2013). Platinum coordination complexes, alkylating agents, and camptothecins are said to also generate high levels of ROS (Wang et al., 2013).

Studies further revealed that two major reasons are attributed to increased ROS levels in chemotherapy; elevation of mitochondria ROS generation and suppression of the cellular antioxidant system (Yen et al., 2012). Antibiotics based chemotherapeutic agents such as anthracyclines, doxorubicin, bleomycin, and platinum coordination complexes, also target the mitochondria and induce cellular ROS generation (Marullo et al., 2013). Studies also indicate that reactive oxygen and nitrogen species play key roles in cancer initiation and survival, by modifying tumor growth through numerous cellular and molecular events such as DNA damage and genome instability (Moldogazieva et al., 2018). Nitric oxide (NO) is a bioactive molecule generated by NO synthases (NOSs) in mammalian cells. By interacting with numerous diverse biomolecules, NO and its derivatives are engaged in a broad range of cellular responses, from neurotransmission to regulation of vascular tone to immune responses and tumorigenesis (Xie
and Huang, 2003). In the majority of cancers, NOS activity is implicated with in increased tumor
growth, malignant transformation, angiogenesis and most notably resistance to apoptosis
(Fukumura et al., 2006; Palmieri et al., 2009). Research pointed out that there is a strong
correlation between the prevalence of tumor cells generating inducible NOS (iNOS; i.e., NOS2)
and shortened survival of patients with advanced skin cancer (Lechner et al., 2005). Inhibition of
NO production by cisplatin in vitro was indicated to act synergistically with reduction in tumor
development and increased sensitivity to cisplatin (Godoy et al., 2012). Several pathways are
believed to be activated after treatment with chemotherapeutics. The PI3K/AKT pathway is
believed to be frequently mutated in human cancers (Mayer and Arteaga, 2016).
Chemotherapeutic agents like cisplatin are believed to trigger cancer cell apoptosis and
autophagy through dose-dependent stimulation of ROS-MAPK signaling (Tang and Grimm,
2004).

PROBLEM STATEMENT AND HYPOTHESIS

As stated earlier in my introduction, the first set of palladium and platinum cyanoximates
was synthesized, characterized and described by (Eddings et al., 2004). Their work highlighted
the in vitro effects of bivalent Pd and Pt cyanoxime complexes with ligand derivatives of
substituted cyan-acetamides. Recently, a new group of cyanoximates were synthesized; platinum
and palladium DECO, (DECO:2-cyano-2-oxiamino-N,N’-diethylaminoacetamide) (Klaus et al.,
2015). The quest to identify the potential effects of the newly synthesized cyanoximates as a
plausible treatment for cancer marked the foundation of my research in Dr. Kim’s Lab.

Firstly, I hypothesize that since the initial cyanoximate used shows cytotoxic effect on
cancer cells in a similar pattern to cisplatin, the current cyanoximate I intend to use will also
have a cytotoxic effect on cancer cell lines. I intend to determine the rate of viability and cytotoxicity using the cell proliferation kit XTT.

Secondly, in order to determine whether the treatments triggered oxidative stress in cancer cells, I measured reactive oxygen species generation after treatment. I hypothesize that cells treated with the cyanoximates will have high reactive oxygen species or high oxidative stress.

Thirdly, cisplatin has been reported to act by causing apoptosis or cell death in the treated cancer cell lines. In this regard I hypothesize that the chemicals to be used will demonstrate apoptosis in the cancer cell lines treated.

Finally, I intend to sequence RNA in order to determine the differentially expressed genes and hopefully correlating the same differentially expressed genes with cisplatin, I believe RNA sequence will give a broad overview to the mechanisms underlying the potentiality of the cyanoximates in question. Overall, I intend to use cisplatin and a known cyanoximate Pt(MCO)$_2$ as my control for the experiment, in order to determine the effectiveness of the newly synthesized cyanoximates. I also intend to decipher the most potent of the two treatment, whether palladium or platinum cyanoximate and whether either or both can be used as a close substitute or replacement to the currently used cisplatin.
MATERIALS AND METHODS

Cell Line and Cell Culture Conditions

Cryopreserved human HeLa S3 cells (ATCC, VA) were thawed in a 37°C water bath. The cell line was cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% penicillin and streptomycin antibiotics solution (Corning, NY). The cultures were then maintained at 37°C in a humidified 5% CO₂/95% air atmosphere.

Treatment

All chemicals tested in the present study were obtained from Dr. Nikolay Gerasimchuk from the Chemistry Department at Missouri State University. Cis-diamminedichloroplatinum (Cisplatin, Sigma-Aldrich), and Bis (2-oxiamino-2-cyano-N-morpholyl-acetamide) platinum (Pt(MCO)₂) (Eddings et al., 2004) were used for control experiments. Additionally, cells with no form of treatment were established as non-treated control. Bis (2-cyano-2-oxiamino-N,N’-diethylaminoacetamide) platinum (Pt(DECO)₂) and Bis (2-cyano-2-oxiamino-N,N’-diethylaminoacetamide) palladium (Pd(DECO)₂) (Klaus et al., 2015) were used as major treatment for the experiments. All these chemicals were dissolved in dimethyl sulfoxide (DMSO, Fischer Scientific.) immediately before use. A DMSO treated control was also prepared.

Cell Viability Assay

The extent of viability of HeLa S3 cells treated with these chemicals was evaluated using XTT assay KIT (Biotium, CA). A total of 10,000 cells/well were seeded on a 96-well plate
(Corning, NY) and incubated at 37°C for 24 hr prior to the treatment with varying doses of these chemicals, ranging from 0.125-1 mM, in quadruplicate for 24 hr. Then, XTT solution containing the XTT activator PMS in a 200:1 ratio was applied to each well, after which the plate was read on an ELx808 Absorbance Microplate Reader (BioTek, VT) for absorbance at A450-A630nm. The absorbance values at 5 hr after XTT treatment were collected.

**Reactive Oxygen Species (ROS) Measurement**

Dihydroethidium (DHE) and Dihydrorhodamine 123 (DHR123, DHR) (Biotium, CA) that measure for superoxide and peroxynitrite levels, respectively, were used for this experiment. This investigation was carried out according to the previously described procedure (Balaiya and Chalam, 2014). To prepare stock solutions, DHE and DHR were initially dissolved in DMSO to get the desired concentration (10 mM stock). On the day of the experiment each stock was diluted to yield a 10 µM working concentration using 1X phosphate buffered saline (PBS). A total of 50,000 cells/well was seeded on a 24-well plate (Corning, NY) and cultured for 24 hr. The cells were then treated with 0.1 mM of chemicals indicated in the section above for 24 hr. On day 3, the cells were initially centrifuged to form a pellet, followed by treatment with freshly prepared ROS indicator dyes of 10 µM concentration. Each tube was treated with 1 mL of the dye solution and incubated for 30 minutes in the dark before analyzing with a flow cytometer (Attune NxT acoustic focusing cytometer, Life Technologies). The excitation and emission wavelength set for detecting oxidized DHE and DHR was 518nm/606nm and 507nm/536nm, respectively.

**Apoptosis Measurement**
Apoptosis measurement was conducted as described by the manufacturer’s protocol (Thermo Fischer Sci., https://bit.ly/2mSZfhR). On day 1 of the experiment, 50,000 cells/well were seeded on a 24-well plate. Subsequently, the cells were treated with 0.1 mM of chemicals for 24 hr. On day 3, the cells were treated with 100 µL of 1X Annexin V binding buffer, 5 µL of Annexin V-APC dye (BD Pharmingen), and 5 µL of Propidium Iodide (BD Pharmingen). The cells were then incubated in the dark for 15 minutes and were subsequently analyzed using Attune NxT acoustic flow cytometer (Life Technologies). Annexin V-APC was excited by a laser line at 650nm, and the resulting emission wavelength of 660nm was recorded. The excitation and emission wavelength of propidium iodide was 533nm and 617nm, respectively.

Total RNA Extraction

On day 1 of the experiment, HeLa cells were grown for 24 hr in a 6-well plate at a density of 1.0x10⁶ cells per well. The cells were then treated either with 1% DMSO (non-treated control group) or 0.5 mM Pt (DECO)2 for 24 hr. RNA extraction was performed on day 3 using a TRIzol protocol (Invitrogen). The total RNA concentration was calculated using the Qubit 3.0 Fluorometer (Thermo Fischer Sci.). Final concentrations of total RNA ranged from 150-250 ng/µL. Two µg of total RNA for each sample was sent to the genome sequencing facility (University of Kansas Medical Centre, KS) for mRNA isolation and cDNA synthesis.

mRNA Isolation and cDNA Synthesis

At the genome sequencing center, Stranded mRNA-Seq was performed using the Illumina NovaSeq 6000 Sequencing System. Quality control on RNA submissions was completed using the Agilent TapeStation 4200 using the RNA ScreenTape Assay kit (Agilent
Technologies 5067-5576). Total RNA (1µg) was used to initiate the library preparation protocol. The total RNA fraction was processed by oligo dT bead capture of mRNA, fragmentation, reverse transcription into cDNA, end repair of cDNA, ligation with the appropriate Unique Dual Index adaptors, strand selection and library amplification by PCR using the Universal Plus mRNA-seq library preparation protocol (NuGEN 0508-08, 0508-32).

Library validation was performed using the D1000 ScreenTape Assay kit (Agilent Technologies 5067-5582) on the Agilent TapeStation 4200. Using the Agilent D1000 assayed library concentration, each library was diluted to ~4 nM and a final library quantification was conducted, in triplicate, using the Roche Lightcycler96 with Fast Start Essential DNA Green Master (Roche 06402712001) and KAPA Library Quant (Illumina) DNA Standards 1-6 (KAPA Biosystems KK4903). Using the qPCR results, RNA-Seq libraries were adjusted to 2.125 nM concentration and pooled for multiplexed sequencing. Pooled libraries were denatured with 0.2 N NaOH (0.04 N final concentration), neutralized with 400 mM Tris-HCl, pH 8.0. A dilution of the pooled libraries to 425 pM was performed in the sample tube, on the instrument followed by onboard clonal clustering of the patterned flow cell using the NovaSeq 6000 S1 Reagent Kit (200 cycle) (Illumina 20012864). A 2x101 cycle sequencing profile with dual index reads was completed using the following sequence profile: Read 1 – 101 cycles x Index Read 1 – 8 cycles x Index Read 2 – 8 cycles x Read 2 – 101 cycles. Following collection, sequence data was converted from .bcl file format to fastq file format using bcl2fastq software and de-multiplexed into individual sequences for data distribution using a secure FTP site or Illumina Base Space for sequence analysis (University of Kansas Medical Center Genomics Core).

Analysis of Sequencing Data
Data from the above-mentioned cDNA sequences were analyzed using Basepair tech, a website created for analyzing sequenced data (www.basepairtech.com). The data obtained from Kansas Medical Genome Center was uploaded to the server using the pipeline RNA-seq. Expression count (STAR) was generated for each data set, which comprised of a quality check to ensure that each file of the sequenced data had good quality reads and the data were interpreted correctly. Next, the reads were aligned to the human reference genome used (human hg19 genome) in order to get a list of differentially expressed genes (DEGs). After obtaining the gene data, genes with a q-value greater than 0.05 were not included in the final list of analyzed genes. The selected genes were grouped based on correlating Gene Ontology (GO) terms obtained from GOrilla.

**Statistical Analysis**

Statistical analysis was carried out with GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Analysis and comparison of data from treatment and control groups was evaluated using one way analysis of variance (ANOVA) and Dunnett’s multiple comparison test. Differences between groups were considered to be significant at a p value of <0.05. Data was represented as represented as represented as Mean ± S.D. A single * represents p < 0.05, ** represents p < 0.02, and *** represents p < 0.01, and **** represents p < 0.001
RESULTS

Pt(DECO)\textsubscript{2} Mediated Reduction in Cell Viability

Little research has been conducted to assess the potential cytotoxic effects of metal cyanoximates on cancer cells, except for a couple of reports illustrating the effects of Pt(MCO)\textsubscript{2} and Pd(MCO)\textsubscript{2} on HeLa cell viability. Those studies employed the use of Trypan Blue assay for cell viability quantification, revealing that the treatment of Pt(MCO)\textsubscript{2} and Pd(MCO)\textsubscript{2} on HeLa and WiDr cells results in decreased cell viability up to 16% and 28%, respectively (Eddings et al., 2004; Ratcliff et al., 2012a). The current study, for the first time, evaluated the effects of Pt(DECO)\textsubscript{2} and Pd(DECO)\textsubscript{2} on HeLa cell viability by measuring the absorbance of XTT tetrazolium at 450-630nm (Fig, 1). HeLa cells were treated with the metal cyanomixates, ranging from 0.125 mM to 1 mM by a two-fold serial dilution. Concurrently, I varied the concentration of DMSO, the vehicle control from 10% to 1.25% in order to accurately assess the actual effect of the tested metal cyanoximates. With 1 mM of Pt(DECO)\textsubscript{2}, Pd(DECO)\textsubscript{2}, and Pt(MCO)\textsubscript{2}, my results showed significant reduction in cell viability extent to which only less than 25% of cells were viable after the treatment (Fig, 1A). However, this marked decrease in viability can be largely attributed to the 10% DMSO used to solubilize each of these metal cyanomixates, on the basis of my observation that 10% DMSO controls also reduced cell viability drastically (Fig, 1A). It was found that with 0.5 mM of cisplatin or Pt (DECO)\textsubscript{2} diluted in a final concentration of 5% DMSO, significantly reduced cell viability when compared with the 5% DMSO vehicle control (Fig, 1B). The mean absorbance of cells treated with 5% DMSO alone was recorded at 0.3985 ± 0.012, significantly different from that of cisplatin (0.119 ± 0.0262, p<0.0001) and that of Pt(DECO)\textsubscript{2} (0.2713 ± 0.060, p=0.004). At 0.25 mM of metal cyanoximates with 2.5% DMSO,
cisplatin caused reduced cell viability with an absorbance value of 0.295 ± 0.05, when compared with 2.5% DMSO alone (0.512 ± 0.177). At the same concentration, Pt(DECO)$_2$ showed modest reduction in cell viability (0.384 ± 0.083), without statistical significance (p=0.325) (Fig. 1C). At the lowest concentration of 0.125 mM with 1.25% DMSO, cisplatin affected cell viability with absorbance value of 0.605 ± 0.124, exhibiting significant difference (p=0.0025) when compared with 1.25% DMSO used as control 0.825 ± 0.059 (Fig. 1D). At the same concentration, none of metal cyanoximate-treated cells showed changes in cell viability. These results led me to the conclusion that Pt(DECO)$_2$ and Pd(DECO)$_2$ are not as effective as cisplatin in reducing cell viability. Notwithstanding, Pt(DECO)$_2$, among those new metal cyanoximates, was most effective to reduce cell viability even with its concentration at 0.25 mM.

**Metal Cyanoximates did not Significantly affect ROS**

Studies have shown a positive correlation between the efficacy of chemotherapeutic anticancer drugs and elevation of reactive oxygen species (ROS) (Gupta et al., 2012; Teppo et al., 2017; Yang et al., 2018). Several ROSs exist, but specifically, nitric oxide, superoxide and peroxynitrite have all been implicated in several cancer pathogeneses (Korde Choudhari et al., 2013) and have also been targeted for cancer treatment (Korde Choudhari et al., 2013; Weinberg et al., 2019). However, there are no studies delineating the effect of metal cyanoximates on ROS alteration. This prompted me to test the potential impact of metal cyanoximates on alteration of ROS levels using two well-known ROS indicators, Dihydrorhodamine (DHR) and Dihydroethidium (DHE) that detects peroxynitrite and superoxide levels, respectively (Dikalov and Harrison, 2014). My results showed that 0.1 mM of cisplatin caused a significant reduction in peroxynitrite levels with the mean percentage of 38.57 ± 2.821 (p<0.0001) when compared
with 1% DMSO control samples with the mean of 75.91 ± 2.821 (Fig 2, A&B). In contrast, cell samples with metal cyanoximates, including Pt(DECO)$_2$, Pd(DECO)$_2$, and Pt(MCO)$_2$ at their 0.1 mM concentration, showed no effects in altering peroxynitrite levels, comparable to DMSO control samples without statistical difference (Fig 2, A&B). Based on my processed data from flow cytometry (Fig 3, A&B), cisplatin at its 0.1 mM concentration significantly elevated superoxide levels, with an average of 32.82% ± 1.293 (p<0.0001). The mean of DMSO treated control stayed at 16.70 ± 5.650, whereas all tested metal cyanoximates caused no alteration in superoxide levels similar to that of DMSO treated control (Fig 3, A&B). These findings suggest that 0.1 mM concentration of Pt(DECO)$_2$ and Pd(DECO)$_2$ do not show significant effects on production of ROS.

**Metal Cyanoximates Induced Apoptosis at Low Concentration**

The metal cyanoximates including Pt(DECO)$_2$ led to reduced cell viability, but not as effective as cisplatin (Fig, 1). Interestingly, however, the treatment with Pt(DECO)$_2$ caused no alteration of ROS levels (Figs, 2&3). In this regard, I hypothesized that the cell viability decrease with these metal cyanoximates may be attributed to significant levels of apoptosis induced by them. To validate my hypothesis, I exploited Annexin V-APC and Propidium Iodide that detect early and late stage of apoptosis, respectively (Lakshmanan and Batra, 2013; Wlodkowic et al., 2009). With 0.1 mM of Pt(DECO)$_2$, Pd(DECO)$_2$, or Pt(MCO)$_2$, my results showed increased levels of early apoptosis as cisplatin (Fig 4, A&B), that was significantly different when compared to DMSO control. However, levels of late apoptosis in treated cells were not distinctly different from control with DMSO, because only less than 1% of cells in treated samples were in
a late stage of apoptosis (Fig. 4C). Elevation of early apoptosis with 0.1 mM of treated cyanoximates reveals that Pt(DEC0)2 and Pd(DEC0)2 can cause programmed cell death.

Figure 1 Measurement of cell viability using an XTT cell viability kit. A total of 10,000 HeLa cells per well were seeded on a 96 well plate. The cells were treated with varying amounts of metal cyanoximates 24hrs after plating. The total treatment time with those metal cyanoximates was 24 hrs prior to the measurement of the tetrazolium absorbance for each sample at A450nm-A630nm. A) The absorbance of treated cells at 1.0 mM concentration. B) The absorbance of
treated cells at 0.5 mM concentration. C) The absorbance measurements at 0.25 mM. D) The absorbance measurements at the lowest treatment of 0.125 mM concentration. A single * represents p < 0.05, ** represents p < 0.02, and *** represents p < 0.01, and **** represents p < 0.001

**Figure 2** Measurement of peroxynitrite levels using DHR. On day 1, 50,000 cells per each well were seeded and cultured on a 24 well plate for 24 hrs. The cells were then treated with 0.1 mM of metal cyanoximates for 24 hrs prior to collecting them for the ROS analysis stated in the Materials and Methods section. Harvested cells were analyzed using the Attune Nxt Flow cytometer at excitation and emission wavelength of 507nm and 536nm, respectively. A) A representative flow cytometer image of each treatment and the corresponding gating used. Three
replicates were used, but only one of the images is displayed. B) Analyzed data after using Prism. Each bar represents the average of three replicates used. A single * represents p < 0.05, ** represents p < 0.02, and *** represents p < 0.01, and **** represents p < 0.001.

**Figure 3** Measurement of superoxide levels using DHE. On the first day of the experiment, seeded 50,000 cells per well onto a 24-well plate. The cells were then treated with metal cyanoximates for 24 hrs. On the third day, the cells were harvested for ROS analysis using the procedure described in the Materials and Method section. The cells were analyzed using Attune Nxt Flow cytometer at excitation and emission wavelength of 518nm and 606nm, respectively. A) A representative flow cytometer image of each treatment and the corresponding histogram gating used. Each treatment has exactly the same gating. Three replicates were used, but only one of the images is displayed. B) Analyzed data from Prism. Each bar represents the average of three replicates used. A single * represents p < 0.05, ** represents p < 0.02, and *** represents p < 0.01, and **** represents p < 0.001.
Figure 4 Measurement of HeLa cell death using Annexin V-APC and Propidium Iodide for early and late apoptosis, respectively. On day 1, 50,000 HeLa cells were seeded per well on a 24 well plate. The cells were then treated with 0.1 mM of metal cyanoximates for 24 hrs prior to collecting them for apoptosis measurements. On day 3, the cells were stained with Propidium Iodide and Annexin V-APC and were measured using a flow cytometer as detailed in the Materials and Method section. A) A representative flow cytometer image of each treatment and the quadrat gating used. The same gating was used for each treatment. The top left part of the quadrat shows necrotic cells, while the bottom left shows live cells. The top right quadrat shows late apoptotic cells, while the bottom right indicates early apoptosis. Three replicates were used, but only one of the images is displayed. B) Analyzed data after using Prism. Each bar represents the average of three replicates used with treatments showing early apoptosis. C) Analyzed data from Prism with treatments showing late apoptosis. A single * represents p < 0.05, ** represents p < 0.02, and *** represents p < 0.01, and **** represents p < 0.001
cDNA Sequencing Reveals Up and Down Regulated Genes with Pt(DECO)₂

Instead of concluding my studies based on data obtained from limited simple methods of assessing cell viability, reactive oxygen species, and apoptosis, I decided to conduct an RNAseq analysis in order to examine the global impact of the cyanoximates on HeLa cells. Due to my results in the previous section that demonstrate the potential of Pt(DECO)₂ in inducing apoptosis, I examined the transcriptomic response in HeLa cells exposed to 0.5 mM concentration of Pt(DECO)₂ by performing next-generation RNA sequencing that generates quantitative gene expression data for both the control and Pt(DECO)₂ treated cells. The rationale for the increased concentration was that 0.1 mM was not sufficient to cause cell viability defects and that 1 mM of it was not possible due to the chemical’s solubility with low amount of DMSO, such as 1%.

Briefly, the control and Pt(DECO)₂ treated cells were subjected to total RNA extraction. Total RNA samples were then shipped to the genome sequencing center for mRNA isolation and conversion to cDNA. Both the control and Pt(DECO)₂ treated samples were tested in triplicate, and the newly synthesized cDNA libraries were sequenced with a next-gen DNA sequencer (Illumina) that generates data set sequence for individual replicate. For differential expression analysis, each cDNA data set was uploaded and processed through the computational analysis platform (www.basepairtech.com), using the pipe line RNA-seq. The first process of analysis is to generate the expression count (STAR) on each data set. After trimming and processing, an average of 35,047,609 accepted reads were gathered from the DMSO (1%) control groups and 35,694,261 from the Pt(DECO)₂ treated groups. Of these pure reads, an average of 97% and 95% of the total reads mapped to the human reference genome used (human hg19 genome) in the control and Pt(DECO)₂ treated samples, respectively.
I identified that the expression levels of 1,703 genes in the Pt(DECO)\textsubscript{2} treated samples were found to be statistically different (q<0.05) when compared to the non-treated control. Of all these significant genes, 869 genes were identified to be upregulated and 834 downregulated.

From the gene pool, I obtained GO terms with GOrilla and found that 72% (627 of 869 genes) of the upregulated genes are responsible for cellular processes including, diffusion, energy production, cell transport, respiration, cell interaction, cell communication, and cell death. Likewise, genes implicated in metabolic processes constitute 51% of the upregulated genes (Fig, 5A). A good number of the upregulated genes are also associated with regulation of gene expression (27%), transport (24%), catabolic processes (17%), translation (10%), protein localization (14%), and RNA processing (9%). In addition, 99, 49, 48, 16, and 3 upregulated genes found by the RNAseq analysis are known to be involved in cell response to stress, cytokine-mediated signaling pathway, protein ubiquitination, electron transport chain, and regulation of intrinsic apoptotic pathway, respectively (Fig, 5A).

The GO term analysis on the downregulated genes reveals that regulation of biological activities is greatly downregulated at (69%) (Fig, 5B). Other processes that were downregulated includes developmental processes (33%), regulation of cell communication (23%), transcription regulation (18%), and cell cycle processes (7%) (Fig, 5B).

To further understand the cellular changes caused by Pt(DECO)\textsubscript{2} treatment, I selected 190 most upregulated and 190 most downregulated genes, these genes have a log2fold change of at least 1.5. The vast majority of the highly upregulated genes are involved in multicellular organismal process (23%), and other highly upregulated process includes cornification or keratinization, regulation of gene expression, and gene silencing (Table 1). In the multicellular organismal process category, SOST and SOX3 genes are 5.4-fold and 5.6-fold upregulated,
respectively. Several histone genes, including HIST1H3A known to be involved in multicellular organismal process, regulation of gene expression, gene silencing, nucleosome assembly, chromatin silencing, and chromatin silencing, are upregulated. HIST1H4B is also implicated in regulation of gene expression, gene silencing, nucleosome assembly, and chromatin silencing (Table 1). My GO term analysis with 190 most downregulated genes showed that 52 out of 190 genes are involved in developmental processes. Other downregulated processes include signaling, regulation of synapse assembly, regulation of multicellular organismal process, and anatomical structure development (Table 2). NODAL (nodal growth differentiation factor gene) and SIX 1 (six homeobox 1 gene) appear to be involved in most of the downregulated processes, and both genes are involved in promoting tumorigenesis and transcription regulation (Kalyan et al., 2017; Xia et al., 2014) (Table 2).
Figure 5 Gene Ontology terms of differentially expressed genes (DEGs) with Pt(DECO)₂. All DEGs were grouped into clusters of GO terms shown in the figure. A total of 1,703 DEGs were found to be statistically significant with q value below 0.05. A) Among 1,703 DEGs, 869 genes were found to be upregulated. B) The quantification of downregulated genes associated with their specific GO terms. Of the 1,703 DEGs, 834 genes were found to be downregulated.
Table 1: Go term analysis with 190 top upregulated genes. A total of 190 upregulated genes with log2fold change of at least 1.5 were selected and analyzed using Gorilla (only 90 genes generated GO terms used in this table).

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<th>Gene Ontology Term</th>
<th>No of Genes</th>
<th>Corresponding genes</th>
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<tbody>
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<td>Multicellular organismal process</td>
<td>46</td>
<td>NPAS4, HIST1H3A, MOV10L1, CHRNA2, BRS3, FOXS1, KCNK3, KRT77, PCDH8, KRT3, SOST, CYP4F11, TRPM8, KRT37, SOX8, CACNA1S, JPH4, STC1, ACTC1, F2RL2, ANGPTL2, ALOX15, KRT34, ARHGDIG, ALK, MYH8, CRYAB, MYH6, KRT5, KRT6A, FGF4, KRT7, PLA2G2A, PPEF2, NRROS, SLC34A2, DAZL, SEZ6L, TRIM54, MYL9, RBM11, KRT72, HIST1H3F, NPTX2, KRT76, IRX4</td>
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<td>Regulation of gene expression</td>
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Table 2: Go term analysis with 190 top downregulated genes. A total of 190 downregulated genes with log2fold change of at least 1.5 were selected and analyzed using GO terms.

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<td>Developmental Process</td>
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<td>Regulation of synapse assembly</td>
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DISCUSSION

Chemotherapy remains one of the major therapeutic approaches treating a wide variety of cancer malignancies (Tiwari et al., 2018). However, development of resistance to chemotherapy treatments and underlying broad spectrum of side effects of modern anti-cancer drugs have warranted a search for novel agents that could effectively work against cancer. The pursuit for finding an alternative metal based anti-cancer agent with limited side effects led to the synthesis and characterization of metal cyanoximates (Eddings et al., 2004; Ratcliff et al., 2012b). Only very few published studies value the prospects of newly prepared cyanoximates, specifically Pt(DECO)$_2$ and Pd(DECO)$_2$ (Eddings et al., 2004; Ratcliff et al., 2012a; Ratcliff et al., 2012b). Therefore, to my knowledge, I am one of the first to investigate their potentials as novel anti-cancer agents. The present study is aimed at exploring the effects of freshly synthesized metal cyanoximates on HeLa cancer cells. The results herein provide valuable data and lay the groundwork for showing the promising effect of metal cyanoximates in cancer chemotherapy.

Effects of Metal Cyanoximates on Cell Viability

One of the approaches that can be exploited for testing the potency of chemical compounds on cancer cells is the use of cell viability and proliferation assays (Adan et al., 2016). For this study, I employed an XTT tetrazolium dye based kit that offers a rapid and precise procedure to estimate cell proliferation and cytotoxicity (Adan et al., 2016). Although the trypan blue assay has been described as a low-cost protocol, the method is associated with a high variance and lack of precision with results (Piccinini et al., 2017). MTT assay uses tetrazolium salt known as 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to measure cell proliferation and cytotoxicity (Jo et al., 2015). The time-consuming washing step of
solubilizing the MTT formazan crystal product in an organic solvent (van Tonder et al., 2015) makes the XTT assay more efficient to use. The present study revealed that Pt(DECO)\(_2\) reduces cell viability, comparable to the decreased level of cell viability caused by cisplatin as shown by my XTT data. Additionally, the findings revealed that Pt(DECO)\(_2\) exhibits stronger anti-cancer functions against HeLa than Pd(DECO)\(_2\) and previously tested Pt(MCO)\(_2\). Pt(DECO)\(_2\) at 0.25mM concentration caused a 13% net reduction in cell viability, whereas Pd(DECO)\(_2\) and Pt(MCO)\(_2\) did not show lessened viability at the same concentration. This finding is consistent with a previous investigation (Ratcliff et al., 2012a) that reported an excellent efficacy of platinum cyanoximates in regards to their activity against cancer cells. The study showed that treatment of 0.1 mM Pt(MCO)\(_2\) diminished HeLa and WiDr cell populations by 17% and 20%. However, the same concentration of Pd(DECO)\(_2\) reduced the cell population by only 5% and 15%, respectively. I attribute the better anti-proliferative effect of Pt(DECO)\(_2\) to the structural flexibility of the chemical, and this flexibility supports its thermodynamically stable state as well as its suitable kinetic disposition that mediates its faster solubility and better anti-cancer effect. The lesser biological activity of other cyanoximates can be linked to the increased bulkiness and rigidity of their anion, and therefore, there is higher energy barrier between the atom structure making it less flexible and more conformational (Personal communication with Dr Gerasimchuk from Missouri State University).

**Effects of Metal Cyanoximates on Reactive Oxygen Species**

One of the mechanisms of action exhibited by chemotherapeutic agents is the generation of ROS to induce cell death (Yang et al., 2018). In the current study, I observed that 0.1mM cisplatin increased superoxide levels, which is in good agreement with previous findings that
cisplatin treatment elevates ROS concentration to levels above the threshold that causes cancer cell death (Chanvorachote et al., 2006; Dasari and Tchounwou, 2014; Schweyer et al., 2004; Yang et al., 2018). On the contrary, I observed that under the same conditions, cisplatin decreased the amount of peroxynitrite in treated HeLa cells. Taking into consideration the fact that the combination of nitric oxide with superoxide anion leads to formation of peroxynitrites (Chanvorachote et al., 2006; Radi, 2018), we conjecture that the decline in peroxynitrites could be due to a reduction in endogenous nitric oxide levels, but not superoxide due to my observation that superoxide levels increased with cisplatin (Fig. 3). This idea is in line with the previous findings that have explained that reduction in the levels of endogenous nitric oxide in melanoma cell lines treated with cisplatin correlated to depletion in cell growth and enhanced cisplatin induced apoptosis (Tang and Grimm, 2004). Likewise, a reduction in nitric oxide levels after cisplatin treatment was linked to improved sensitivity and better response to cisplatin, while elevated nitric oxide levels promoted cisplatin resistance (Godoy et al., 2012; Leung et al., 2008). As opposed to cisplatin, the newly tested metal cyanoximates did not reveal significant alterations in both nitric oxide and superoxide levels, and I therefore, conclude that this may be due to a milder effect of the novel compounds compared to cisplatin at the same conditions. Moreover, several kinds of ROS exist (Li et al., 2016), and I am of the opinion that metal-cyanoximates may have a more noticeable impact on other types of ROS. At the same time, I cannot exclude the possibility that no alteration of ROS levels with Pt (DECO)₂ could be due to a slight difference in mode of action between cisplatin and the tested metal cyanoximates.

**Metal Cyanoximates and Apoptosis in HeLa cells**
Cisplatin, Carboplatin and Oxaliplatin are all platinum complexes currently used in multiple cancer treatments (Dasari and Tchounwou, 2014; Mikula-Pietrasik et al., 2019; Ndagi et al., 2017). The present study showed that all metal cyanoximates induce apoptosis in treated HeLa cells at 0.1 mM concentration. I postulate that tested Pt(DECO)$_2$, Pd(DECO)$_2$ and Pt(MCO)$_2$ promoted apoptosis using a mechanism similar to currently used chemotherapeutics. The most commonly described mechanism supported by a growing body of evidence is that most anti-cancer drugs mediate apoptosis through the intrinsic mitochondria-dependent apoptotic pathway (Pistritto et al., 2016). This pathway is believed to be initiated in response to intracellular stress signals such as DNA damage and excess ROS and is observed in most chemotherapy treated cells (Ricci and Zong, 2006). Studies have also revealed that metal based chemotherapeutic agents form covalent bonds with DNA, halting cell division and consequently inducing apoptosis (Dasari and Tchounwou, 2014; Ndagi et al., 2017). In order to understand the precise mode of action for the newly studied metal cyanoximates, I present a model below that contains highly upregulated and downregulated processes induced after Pt(DECO)$_2$ treatment.

**Upregulated Gene Expressions Mediated by Pt(DECO)$_2$ Treatment and their Proposed Mechanism of Action**

Here, I provide a model (Fig, 6A&B) depicting the key upregulated genes and their corresponding cellular functions. From the model illustrated in Figure 6A, I highlighted the major processes upregulated after Pt(DECO)$_2$ treatment. These processes include DNA damage response, p53 activation, cell cycle check point, and apoptosis (Fig, 6A). PARP3 is known to be activated as a result of DNA damage response (Sousa et al., 2012). With the upregulation of PARP3 in my RNAseq analysis (Fig, 6A), one can propose that Pt(DECO)$_2$ treatment may cause significant DNA damage in HeLa cells. It has been known that the modulation of p53 activity
plays a key role in DNA damage response (Helton and Chen, 2007). Interestingly, my RNAseq analysis revealed that DAXX gene (death domain-associated protein), a well-known p53 regulator (Brazina et al., 2015), was significantly upregulated. Further, a study has shown that DAXX binds to MDM2 (mouse double minute2 homolog), concurrently with Hausp (herpesvirus-associated ubiquitin-specific protease; also known as USP7) to stabilize the activity of MDM2 to modulate p53 function (Tang et al., 2010; Tang et al., 2006). When cells are damaged or exposed to DNA damaging agents, the MDM2-DAXX interaction is distorted in an ATM-dependent manner, resulting in p53 activation (Tang et al., 2013; Tang et al., 2006). Though the DNA damage induced by the treatment of Pt(DECO)$_2$ in my experiment upregulates DAXX expression, the elevated DAXX may not secure MDM2 activity in this scenario, rather leading to hyper activation of p53 for cell death.

DNA damage is one of the prerequisite factors that promote cell cycle checkpoint system to slow down or halt the progression of cell division (Visconti et al., 2016). As cells enter a quiescent stage (G0), levels of GAS7 (Growth arrest specific 7) expression are elevated (Ju et al., 1998). I found that Pt(DECO)$_2$ treatment led to increased GAS7 expression (Fig, 6A), suggesting that the treated cells are less likely proliferative. This was indeed the case proven by my XTT assay results (Fig, 1). The level of GAS7 is also implicated in regulating the severity of metastasis. Studies have demonstrated that lower level of GAS7 expression promotes tumor metastasis in several cancer types (Chang et al., 2018; Li et al., 2017a; Tseng et al., 2015). However, increased expression of GAS7 gene has been observed in Oxaliplatin-treated human hepatocellular carcinoma cells where it mediated proliferation reduction (Li et al., 2017a). From these findings, I can infer that Pt(DECO)$_2$-mediated GAS7 upregulation leads to the decreased cell proliferation.
Elevated activity of p53 is associated with cell cycle arrest and apoptosis (Chen, 2016). In agreement with this, the RNAseq analysis revealed two highly expressed pro-apoptotic genes, CASP4 (caspase 4) and AIFM2 (apoptosis-inducing factor, mitochondrion-associated 2) (Fig, 6A). The former is shown to be upregulated in response to endoplasmic reticulum stress induced via an increased number of unfolded and misfolded proteins (Limonta et al., 2019; Bian et al., 2009; Shibamoto et al., 2017). Additionally, a recent study showed that decreased CASP4 expression is associated with poor prognosis of esophageal squamous cell carcinoma. This finding supports the notion that CASP4 promotes inflammatory and immune response that may inhibit squamous cell carcinoma progression (Shibamoto et al., 2017). AIFM2 has been associated with mitochondria-mediated apoptosis signaling (Miriyala et al., 2016), and its upregulation promotes apoptosis in lung cancer cells (Lu et al., 2016). CASP4 or AIFM2 can serve as an upstream apoptotic mediator to enhance CASP9 activation (Mbuthia et al., 2017; Yamamuro et al., 2010) and that Caspase 9 activation subsequently initiates Caspase-3 activation, which eventually activates the rest of the caspase cascade and promotes apoptosis (Ghobrial et al., 2005). Taken together, I propose that Pt(DECO)2 treatment stimulates apoptosis in a caspase dependent manner.

**Downregulated Gene Expressions Mediated by Pt(DECO)2 Treatment and their Proposed Mechanism of Action**

Based on the GO terms list generated from GOrilla, multiple highly downregulated genes were identified in response to Pt(DECO)2 treatment. The most downregulated genes were responsible for drug transport, cell cycle progression, transcription regulation, proliferation, super elongation complex and anti-apoptosis (Fig, 6B). SLC47A2, SLC1A3, SLC16A9, ABCA1,
ABCA3, and ABCA12 are all classified as drug transporters and are downregulated in the model (Fig, 6B). Drug transporters in cells are classified into solute carriers (SLC), and ATP-binding cassettes (ABC) transporters (Li and Shu, 2014; Schumann et al., 2020). Evidence has shown that cancer cells utilize the effect of SLC to promote growth and survival (Li and Shu, 2014; Lin et al., 2015). ABC transporters are efflux drug transporters that utilize energy from ATP hydrolysis to pump toxic drugs out against their concentration gradient. Increased expression of several ABC transporter genes are associated with reduced cellular accumulation of anticancer agents and acquired drug resistance in treated cell lines (Li and Shu, 2014; Schumann et al., 2020). Thus, Pt(DECO)$_2$ treatment enhances the downregulation of drug transporter genes that may otherwise induce drug resistance in treated cancer cells.

SIX1 and SIX4 are members of Sine Oculis Homeobox (SIX) homolog family that has historically been implicated in regulating transcription. Their upregulation is thought to result in tumor metastasis, growth, and invasion in non-small cell lung cancer (Xia et al., 2014). In contrast, the present result of downregulation of SIX1 and SIX4 by Pt(DECO)$_2$ (Li et al., 2017b), is suggestive of a role in decreasing cancer proliferation.

NODAL gene is a member of the TGF-β family of proteins that is essential for development of embryonic cells (Kalyan et al., 2017; Strizzi et al., 2012). Recently, findings have elucidated that NODAL gene is highly expressed in multiple cancer types and functions by promoting carcinogenesis (Kalyan et al., 2017; Strizzi et al., 2012). The model displayed that Pt(DECO)$_2$ treatment reduced the expression of NODAL gene as illustrated in Fig 6B. Downregulation of NODAL gene provides evidence that Pt(DECO)$_2$ fosters the reduction of viability in cancer cells by suppressing the genes that enhances rapid growth and proliferation.
Furthermore, BCL2, a well-known anti-apoptotic gene commonly overexpressed in several cancer types was also found to be downregulated (Frenzel et al., 2009). Notably, the anti-apoptotic potential of BCL2 has also been correlated to chemotherapy resistance, with findings suggesting that overexpression of this genes makes it rather difficult to eliminate malignant cells with cytotoxic anticancer agents (Reed, 2018). Downregulation of BCL2 genes by Pt(DECO)₂ further supports its role in promoting apoptosis. I also noticed the downregulation of a key component of the super elongation complex (SEC), AFF4 gene (Fig, 6B). The elongation stage of transcription is regulated by a complex group of genes that compromise the super elongation complex (SEC). The product of the AFF4 gene directly binds with P-TEFb and AF9 or ENL and is needed for the formation of SEC (Luo et al., 2012a; Luo et al., 2012b). Overexpression of the AFF4 gene has been shown to promote cell growth and proliferation in head and neck cell squamous cell carcinoma (HNSCC) (Deng et al., 2018). The model revealed that AFF4 gene was downregulated, implying that Pt(DECO)₂ can help reduce cancer cell proliferation.

Genes that maintain the cell cycle have been shown to promote rapid growth and proliferation in cancer cells (Feitelson et al., 2015). The model presented indicates that GTSE1, ESCO2, NPAT, and E2F8 genes were downregulated (Fig, 6B). E2F8 is a key member of the E2F family of transcription factors that have demonstrated key roles in cell growth, differentiation and cycle regulation (Lv et al., 2017). E2F8 over expression has been noted in hepatocellular carcinoma (HCC) affirming its role in tumorigenesis (Baiz et al., 2014). Likewise, GTSE1 is upregulated in several human cancers owing to the fact that it negatively regulates p53 expression (Guo et al., 2016; Spanswick et al., 2012). A recent study, however, established that GTSE1 overexpression in hepatocellular carcinoma (HCC) promoted the metastasis and proliferation in the cell line (Guo et al., 2016). Both GTSE1 and E2F8 were downregulated in the
model, declining their functions in HeLa cells treated with Pt(DECO)$_2$. We deduced from the presented model that Pt(DECO)$_2$ suppresses activities of drug transporters, anti-apoptotic genes, genes that promotes proliferation as well as genes that represses apoptosis. The overall findings highlight the roles of Pt(DECO)$_2$ as a potential anti-cancer agent through different activities of differentially expressed genes and their several roles in reducing cell proliferation and promoting apoptosis.
Figure 6 Schematic models of the proposed physiological changes with Pt(Deco)\textsubscript{2} treatment in HeLa cells. All genes in yellow rectangles in the models are either upregulated or downregulated according to my RNAseq analysis. A) Pt(Deco)\textsubscript{2} appears to upregulate genes responsible for cornification, nucleosome assembly, DNA damage response, p53 modulation, cytochrome activities in the ER, metabolism in the mitochondria, cell cycle control, and apoptosis. From all these processes, we selectively described the following cellular processes in the Discussion section: DNA damage response, p53 modulation, cell cycle control, and apoptosis. B) Several genes appear to be downregulated by the presence of Pt(Deco)\textsubscript{2}, including genes involved in drug transport, glutamate binding, transcription regulation, cell cycle progression, electron transport chain, mitochondria metabolism, anti-apoptosis, super elongation complex, DNA repair, and cell proliferation. We chose to provide more in-depth explanation on the following cellular processes in the discussion section: drug transport, cell cycle progression, transcription regulation, proliferation, super elongation complex and anti-apoptosis.
CONCLUSION

In the present study, I assessed the anti-cancer potentials of newly synthesized metal cyanoximates on HeLa cells, and provided evidence that treatment with metal cyanoximates enhanced reduced viability and apoptosis. Furthermore, I selected Pt(DECO)$_2$ as the most effective in the group and performed RNA sequence analysis to investigate its mode of action. Differential expression of several hundred genes that play key roles in cell division, cell cycle control, proliferation and apoptosis supported my initial studies that Pt(DECO)$_2$ augments cell death. These novel findings lead me to conclude that Pt(DECO)$_2$ can serve as a potential option for use in cancer chemotherapy.
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