CHARACTERIZATION OF THE PT(II)-INDUCED NUCLEOLAR STRESS RESPONSE PATHWAY

by

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A THESIS

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There are three FDA-approved platinum chemotherapeutics: cisplatin, carboplatin, and oxaliplatin. Until recently, it was thought that all three Pt(II) drugs work through the same mechanism of action, in which the Pt(II) compounds form inter- and intra-strand DNA crosslinks, leading to the activation of the DNA Damage Response (DDR) pathway. However, recent studies have demonstrated that while cisplatin and carboplatin primarily act through the DDR pathway, oxaliplatin may instead elicit its anticancer effects through disruptions to the cellular nucleolus and ribosome biogenesis inhibition. The nucleolus is a membraneless organelle found within the nucleus and primarily functions as the house of ribosome biogenesis. Any disturbances to the nucleolus or ribosome biogenesis causes a cellular response referred to as nucleolar stress. While the DDR pathway is primarily activated by DNA damage, there are a variety of different stimuli that can cause nucleolar stress induction. Examples of nucleolar stress-inducing agents include small molecule compounds Actinomycin D (ActD), BMH-21, and CX-5461, which were used in this research as comparative tools to elucidate characteristics of the Pt(II)-induced nucleolar stress response pathway. Results from these studies demonstrate that Pt(II)-induced nucleolar stress is irreversible, while stress induction by ActD, BMH-21, and CX-5461 is reversible upon drug removal. This finding indicates potential differences in mechanisms between these nucleolar stress-inducing small molecules and Pt(II) compounds. Furthermore, the

cell cycle plays important roles in the nucleolus and is involved in both the nucleolar stress response and DDR pathways. Checkpoint kinase 1 (Chk1) is a regulatory protein involved in various cellular pathways including the cell cycle. Inhibiting Chk1 causes cell cycle arrest in the G_1 phase and prevents activation of the DDR pathway, which primarily occurs in the S phase of the cell cycle. Observing the effect of platinum chemotherapeutic treatments with a Chk1 inhibition provides further insight into the relationship between cell cycle progression, DDR and nucleolar stress. Results from these studies indicate an increase in nucleolar stress induction with cisplatin treatment when Chk1 is inhibited and cells are arrested in the G_1 phase. This is not observed with cisplatin treatment under regular cell cycle progression and indicates that the Pt(II)-induced nucleolar stress response pathway may be cell cycle dependent and involve Chk1 and G_1 cell cycle arrest. Together, this research contributes to the further characterization of the Pt(II)-induced nucleolar stress response pathway, but future works are required to fully understand this unique pathway and the mechanisms of platinum chemotherapeutics.

Acknowledgements

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Glossary

Ribosome: a cellular machine that is composed of RNA and protein and synthesizes proteins in a cell.

Nucleus: a membrane enclosed organelle within the cell that contains the genetic information of the cell in the form of chromosomes composed of DNA and other proteins.

Nucleolus: a small membraneless organelle within the nucleus that is the site of ribosome biogenesis and plays roles in maintaining cellular homeostasis.

Nucleoplasm: the interior space of the nucleus that holds the genetic material of the cell.

DNA Damage Response: one of the two cell death mechanisms being investigated in this thesis. The DNA Damage Response pathway (DDR) is activated upon damage to cellular DNA and works to either repair the damaged DNA or induce cell cycle arrest or cell death via apoptosis.

Nucleolar Stress: also referred to as ribosome biogenesis stress or nucleolar stress-induced apoptosis. Nucleolar stress is the other programmed cell death pathway being investigated in these studies and is induced by disruptions to the nucleolus and ribosome biogenesis.

Checkpoint 1 Kinase (Chk1): within the cell cycle, there are proteins called checkpoint kinases that facilitate the transition of one phase of the cell cycle to next and are involved in various cellular pathways. Checkpoint 1 Kinase manages the transition from the G_1 phase to the S phase.

Nucleophosmin 1 (NPM1): a nucleolar protein located within the nucleolus that is involved ribosome biogenesis and various other cellular processes. This protein relocalizes from the nucleolus into the nucleoplasm during nucleolar stress and can be used to determine nucleolar stress induction.

RNA Polymerase I (RNA Pol I): a protein located in the nucleolus that, along with other transcription machinery, transcribes ribosomal DNA (rDNA) to ribosomal RNA (rRNA)

Topoisomerase II: a protein in the nucleus that manages the tangles and supercoils of DNA during DNA replication.

Introduction

The FDA approved chemotherapeutic agents, cisplatin, carboplatin and oxaliplatin are widely used, yet their mechanisms of action are not well understood.[1,2] These drugs have been used as treatments for various types of cancers since the 1970's, but despite their common use as frontline treatments, the clinical applications of these drugs are often limited by severe side effects and the emergence of drug-resistant cancers. Each of these three platinum-based drugs have been shown to have distinct antitumor activity profiles. For example, many colorectal cancer lines are resistant to treatment with cisplatin and carboplatin but are highly sensitive to treatment with oxaliplatin. The mechanisms that are responsible for the differences in anti-tumor activity between platinum compounds are not well understood. Gaining a better understanding of the mechanisms of these compounds is crucial for the development of more effective clinical treatments that can target drug-resistant cancer lines and produce less severe side-effects.[3–5]

The ability of small molecule platinum compounds to crosslink DNA and trigger the DNA damage response (DDR) pathway is the most well-established mechanism to date.[6–8] DDR is a complex cellular pathway involving various proteins that work to repair DNA damage. Upon DNA damage, two main protein kinases, Ataxia-telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 (ATR), initiate the DDR pathway. The activation of these proteins triggers a cascade of protein activity, including the phosphorylation of the signaling protein H2AX. If the DNA can't be repaired, the cell will often go into cell cycle arrest, by the downstream inhibition of Chk1 or Chk2, or apoptosis by downstream activation of the tumor suppressor protein p53. Recent studies revealed that while cisplatin does act through DDR, oxaliplatin instead induces cell death by inhibiting ribosome biogenesis.[9] Ribosome biogenesis is the process by which ribosomes are made and occurs primarily in the cellular nucleolus. The

mechanism by which oxaliplatin inhibits this process is not well understood and the interactions of platinum drugs in the nucleolus is an area of high interest in the field.[10,11]

Recent studies aiming at improving the understanding of oxaliplatin's mechanism have been primarily focused on the compound's ability to induce ribosome biogenesis inhibition rather than or in addition to DDR.[9,12] oxaliplatin's ability to cause inhibition of ribosome biogenesis at clinically relevant concentrations was determined 15 years ago, when it was shown by Burger et al. to inhibit the production of the 47S rRNA precursor at concentrations as low as 3 μM in the human sarcoma cell line 2FTGH. In this study, cisplatin was also tested but had to be used at 50 μM concentrations or higher to induce the same levels of inhibition as oxaliplatin.[13] Drugs that inhibit ribosome biogenesis have been used to treat cancer since as early as 1954, with the use of the anticancer drug actinomycin D (ActD).[14]

Ribosome biogenesis is the process by which ribosomal DNA (rDNA) is transcribed into ribosomal RNA (rRNA) by RNA Polymerase I (Pol I) and then further processed into ribosomal subunits, which eventually become mature ribosomes.[15] This process takes place in the cellular nucleolus, a sub-compartment of the nucleus composed of DNA, RNA and proteins. The nucleolus has recently garnered interest as an organelle in the last decade, as it has been shown to not only be involved in ribosome biogenesis but also to have other cellular functions in regulating cell cycle and cellular homeostasis.[16,17] Disruption to the integrity or function of the nucleolus is referred to as nucleolar stress.

The DeRose lab is interested in investigating the mechanism of Pt(II)-induced nucleolar stress and in previous works synthesized a variety of Pt(II) derivatives and tested them for their ability to induced nucleolar stress. In these studies, nucleolar stress induction was determined by the relocalization of the nucleolar protein nucleophosmin (NPM1) from the nucleolus to the

nucleoplasm. To quantify NPM1 relocalization, cell images were used calculated the coefficient of variation (CV) of pixel intensity, where lower CV values indicate more extensive relocalization and nucleolar stress induction. Actinomycin D (ActD) was used as a positive control for nucleolar stress in these studies.[11,18,19]

Previous research conducted by the DeRose lab has investigated the nucleolar stress pathway induction of oxaliplatin and other Pt(II) compound analogs synthesized by the research group. Their work found that small differences in the platinum-based compound structure and aromaticity had specific and significant effect on nucleolar stress induction.[11] It was also found that the nucleolar stress response is time-dependent[19] and not correlated with whole-cell or nuclear drug uptake, or platinum DNA adduct formation.[20,21] Furthermore, rRNA transcription inhibition was found to occur shortly after treatment with platinum based chemotherapeutic drug and occurs alongside the nucleolar stress response.[18] Recent studies have shown that with inhibition of ATM or ATR, the rRNA transcription inhibition caused by oxaliplatin treatment decreased in cancer cells, suggesting that the two proteins play a role in the nucleolar stress response pathway. However the rRNA transcription inhibition by oxaliplatin was not completely halted, as there was still significant inhibition of transcription by oxaliplatin which could indicate that while ATM and ATR may play a role in the nucleolar stress response pathway, it is not the sole factor in initiating the pathway.[22] Another recent study suggests that the Pt(II) induced nucleolar stress response could be the result of liquid-liquid demixing of the nucleoli that occurs upon oxaliplatin treatment which was shown to lead to rRNA transcription inhibition and eventually, cell death.[23]

Furthermore, in an effort to investigate if stress-inducing Pt(II) compounds also caused DDR, the DeRose lab examined the phosphorylation of H2AX, a key DDR protein[24] and ATM

and ATR, which are critical in the initial detection of DNA damage and the initiation of DDR. Using a γH2AX imaging assay, the number of positive nuclei for γH2AX was determined at 24 hr. drug treatment time in A549 and U-2 OS cell lines. Significantly higher levels of percent positive γH2AX nuclei for cells treated with cisplatin compared to oxaliplatin and other stress-inducing Pt(II) compounds in both cell lines were observed. Furthermore, ATM activation levels were observed to be only slightly higher than no treatment controls for nucleolar stress-inducing compounds. This indicates that cisplatin causes higher activation of the DDR pathway compared to oxaliplatin and its derivatives.[25] Further experiments investigating phosphorylation of ATM and ATR in addition to inhibition of these proteins suggested that nucleolar stress-inducing compounds interact differently with ATM and cause a distinct phosphorylation profile compared to DDR-inducing Pt(II) compounds.[25]

In this investigation, comparison experiments were conducted between platinum-based compounds and small-molecule nucleolar stress-inducing compounds to characterize the nucleolar stress response, specifically the reversibility of the response. Since the mechanisms of small-molecule nucleolar stress-inducing compounds have been better characterized, a comparison between the platinum based compounds and the small-molecule inhibitors will allow us to further characterize the mechanism of the platinum based compounds and inform future investigations to elucidate the mechanisms.[26]Cell growth and division is highly regulated by various checkpoints to ensure cells meet the necessary conditions to progress through each phase of the cell cycle. Checkpoint kinase 1 (Chk1) plays an important role in the cell cycle as it allows cells to progress from the G_1 phase to the S phase. This protein is also involved in various cellular pathways, including DDR.[10] To investigate the role of cell cycle phases on nucleolar stress, Chk1 was inhibited by treatment with caffeine prior to Pt(II) compound treatment. I aimed

to determine if Pt(II)-induced stress would still occur while cells were arrested in the G_1 phase of the cell cycle. Caffeine was found to be a Chk1 inhibitor in by Cortez et al. and will allow us to observe the nucleolar morphology of cells and the cell response to the drugs.[27]

The differing responses induced by Pt(II) compounds that cause nucleolar stress or DDR have clinical implications as they are known to have varied treatment and side-effect profiles, as well as differences in cell death mechanisms. For example, oxaliplatin is known to cause immunogenic cell death, while cisplatin does not.[28–30] Further study is warranted to provide clarification on the molecular mechanisms by which these compounds induce different responses in cells. Recent works suggest that ATM and ATR may be involved in Pt(II)-induced nucleolar stress.[22] However, our current studies suggest that Pt(II)-induced nucleolar stress may not be primarily involved with ATM and ATR and instead work independently of the DDR signaling proteins.[19,25] More work is needed to fully understand the nucleolar stress response pathway induced by Pt(II) compounds, and further elucidate the mechanisms of action of oxaliplatin and other platinum-based chemotherapeutics.

Methods

Cell Culture

Cell line and culture conditions

In this investigation, A549 human lung carcinoma cells and U-2 OS human osteosarcoma cells were used in cell studies. The cells were cultured at 37 °C, 5% CO_2, in Dulbecco's Modified Eagle Medium (DMEM) and Mccoy's 5A Medium respectively. Both media were supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic. The cell lines were maintained for 11 to 30 passages, with cell splitting occurring every 2-6 days when cells reached 80% confluence. Cell medium was replaced every 2-3 days.

Cell Seeding protocol

Cells were seeded and grown on glass coverslips (Ted Pella product no. 260368, round glass coverslips, 10 mm diam. 0.16–0.19 mm thick) according to previously described methods[11].

Drug treatment conditions

Treatments were conducted on cells at 70-80% confluence. Platinum compound treatments were conducted at 10 μM, Actinomycin D treatments were conducted at 5 nM, BMH-21 treatments were conducted at 3 μM and CX-5461 treatments were conducted at 0.2 μM unless a different concentration was specified. Actinomycin, BMH-21 and CX-5461 stock solutions were frozen in DMSO for storage and thawed upon use. Platinum compounds were made into 5 mM stocks on the day of treatment from solids in water. All stock solutions were diluted in cell medium prior to treatment. Inhibitor compounds were added to cells 12 hours prior to drug treatment and remained for the entirety of the treatment.

Chk1 Inhibitor Conditions

Caffeine (Chk1 inhibitor) treatment was conducted at 1 mM in drug free cell medium. Drug treatments during Chk1 Inhibition experiments were diluted using cell medium with 1 mM caffeine. 100 mM Caffeine stock solutions were made in water and heated to 95 °C to ensure solubility prior to treatment.

Reversibility experiment protocol/conditions

For reversibility experiments, cells were washed three times with phosphate buffered saline (PBS) prior to addition of drug-free cell medium.

Immunofluorescence Assay

NPM1 protocol/conditions

After drug treatment was complete, cells were washed with PBS three times and fixed with 4% paraformaldehyde (PFA) diluted in PBS for 20 minutes at room temperature. PFA was aspirated off and cells were permeabilized with 0.5% Triton-X in PBS for 20 min at room temperature. Blocking steps were then performed with 1% bovine serum albumin (BSA) in phosphate buffered saline with 0.1% Tween-20 detergent (PBST) in two washes for 10 minutes each. Cells were incubated for one hour in the primary antibody for NPM1 (FC-61881, Thermo Fisher, 1:500 dilution in PBST with 1% BSA) and then washed three times with PBST and incubated for 1 hour in secondary antibody Goat Anti-Mouse IGG H&L Alexa Fluor 488 (ab150113, Abcam, 1:1000 dilution in PBST with 1% BSA) and washed three times in PBST. The coverslips were then mounted on slides with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher) according to manufacturer's instructions.

γH2AX protocol/conditions

Repeated the NPM1 protocol except using the primary antibody for γH2AX (CR55T33, Thermo Fisher, 2.5 µg in PBST with 1% BSA) instead of the NPM1 primary antibody.

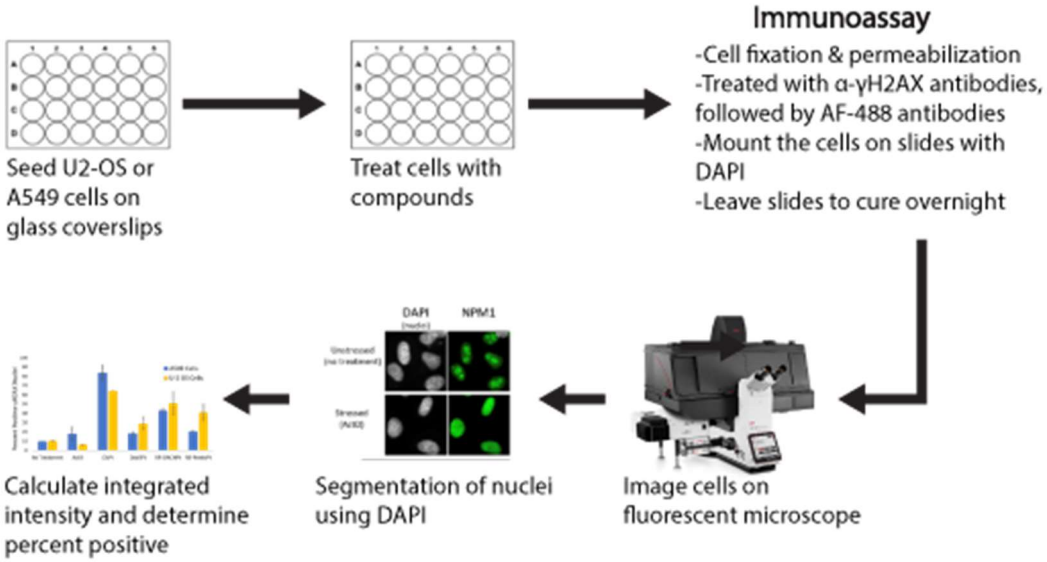

Figure 1: γH2AX Immunofluorescence Assay Workflow

Outline of the workflow of the experiments to determine the extent of activation of the DDR pathway through quantification of γH2AX, a marker for DDR. Fluorescent microscope image from leica-microsystems.com

Chk1 Inhibition protocol

First, the cells were subjected to caffeine treatment at 1 mM. The cells were then washed three times with PBS prior to treatment with platinum compounds and small molecule compounds as described in the Drug Treatment Conditions subsection above. The cells were then subjected to the NPM1 protocol as described above.

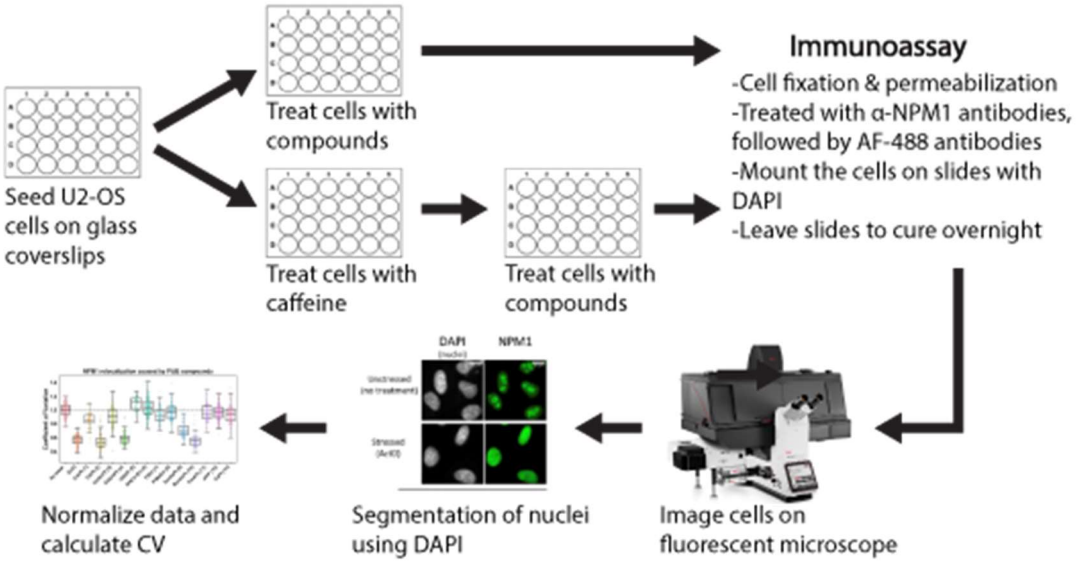

Figure 2: Chk1 Inhibition Immunofluorescence Assay Workflow

Outline of the workflow of the experiments to determine the effect of cell cycle inhibition on NS activation through quantification of NPM1. Fluorescent microscope image from leica-microsystems.com

Nucleolar Stress Reversibility protocol

Following drug treatment, the cells were washed three times with PBS prior to the addition of drug free cell culture medium as the chase step. The cells were then subjected to the NPM1 protocol as described above.

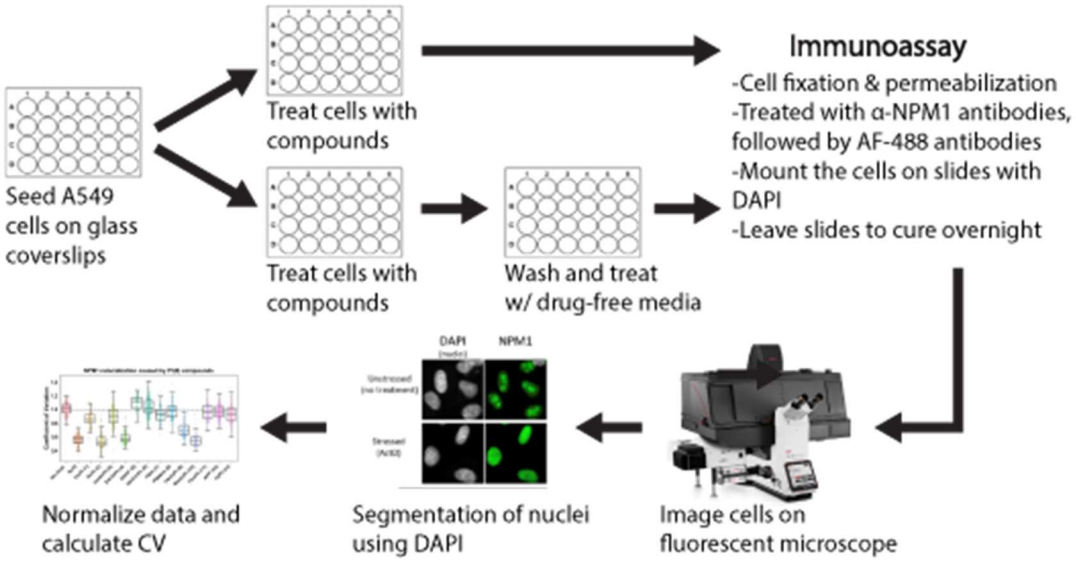

Figure 3: NS reversibility Immunofluorescence Assay Workflow

Outline of the workflow of the experiments to determine the reversibility of the nucleolar stress response in A549 cells through quantification of NPM1. Fluorescent microscope image from leica-microsystems.com

Imaging

Microscope type and fluorescent channels

Images were taken using a HC PL Fluotar 63x/1.3 oil objective mounted on a Leica DMi8 fluorescence microscope using the Leica Application Suite X software. Between 50 and 200 cells were analyzed for each trial and treatment group. The laser intensity and camera exposure were adjusted for each experiment to maximize image resolution.

Quantification

NPM1 Relocalization

NPM1 relocalization was quantified using an analysis pipeline. The images were preprocessed in ImageJ to convert the DAPI and NPM1 channels into separate 16-bit grayscale images. The converted images were then processed using a Python 3 script to segment the nuclei

using the DAPI channel images via Li thresholding functions in the Scikit-Image Python Package[31]. Using the Scikit-Image Python Package, the standard deviation in pixel intensity divided by the mean pixel intensity of each nuclei was calculated and defined as the coefficient of variation (CV). The calculated values were then normalized to the no-treatment control trials of the respective experiments. NPM1 imaging results for each complex were conducted in triplicate trials. The individual CV values for each trial were displayed in boxplots generated using the Seaborn library in Python.

γH2AX Nuclear Intensity

γH2AX quantification was performed using CellProfiler 4.2.1 software, where cells were first segment by nuclei using the DAPI channel and then the integrated intensity for the γH2AX channel was quantified[20]. A percent positive value was calculated for each treatment condition based on the γH2AX intensity values of the untreated control, where all cells with integrated intensities higher than the 90th percentile intensity of the untreated control were defined as positive for γH2AX.

Results and Discussion

Nucleolar stress reversibility and its distinct differences between Pt(II) chemotherapeutic compounds and small molecule inhibitors

To elucidate the Pt(II)-induced nucleolar stress response pathway further, comparative experiments between Pt(II) chemotherapeutic compounds and small molecule RNA transcription inhibitors that induce nucleolar stress were conducted. ActD is known to induce nucleolar stress and has thus been used as a positive control for nucleolar stress in previous experiments.[32] Two other small molecules were chosen for their similar apoptotic outcomes, but different proposed mechanisms. BMH-21 and CX-5461 have both been shown to induce nucleolar stress in multiple cell lines[33,34], but the exact mechanisms of nucleolar stress induction for ActD, BMH-21 and CX-5461 have not been determined and are hypothesized to vary between cell lines.[35–37]

Both ActD and BMH-21 are believed to be DNA intercalators, which interacting with Guanine and Cytosine rich regions of DNA and preventing transcription through their effect on specific molecular machinery that participate in transcription.[14] ActD is considered to inhibit RNA and DNA transcription, in addition to protein synthesis.[38] At lower concentrations, ActD has a more focused effect that specifically inhibits rRNA synthesis.[32] BMH-21 differs in that it has been shown to target RNA Pol I and inhibit its binding to rDNA, preventing formation of the rRNA transcription complex.[26] There is also evidence that BMH-21 induces proteasome-dependent destruction of RPA194, which is the large catalytic subunit of RNA Pol I.[34] This was found to lead to a halting of the polymerase during transcription elongation, revealing a regulatory checkpoint that monitors transcription by Pol I that is sensitized and activated by small molecule BMH-21 and other similar chemotherapeutics.[39] The mechanism of action of CX-5461 remains a topic of debate among researchers. However, multiple studies conducted

under a range of conditions have indicated that CX-5461 may have three cell death inducing pathways. Bruno et al. propose that CX-5461 may be a topoisomerase II poison.[40] The most recent research shows that it may inhibit rRNA transcription by preventing the transcription initiation complex for RNA Pol I.[41–43] Other researchers speculate that is p53-mediated DDR activator.[44]

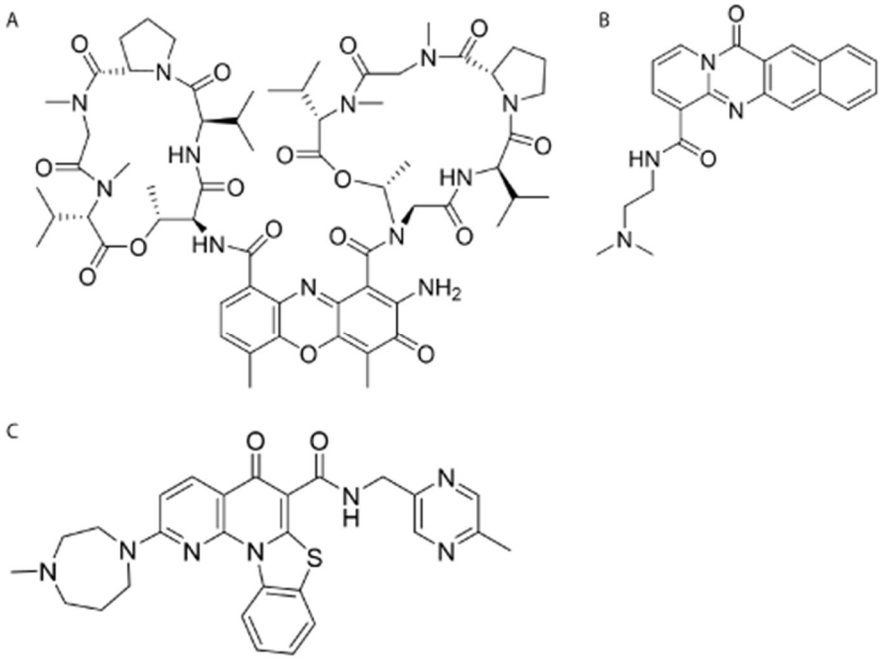

Figure 4: Molecular structures of small molecule RNA Pol I inhibitor compounds

A: Actinomycin D (ActD), B: BMH-21, C: CX-5461

In comparison, nucleolar stress-inducing Pt(II) compounds such as oxaliplatin are chemically distinct from the small molecule nucleolar stress-inducing compounds as seen in Figure 4 in comparison to the structure in Figure 5 below.

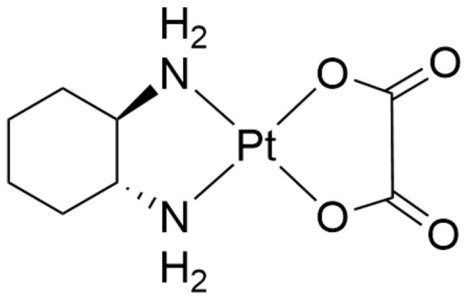

Figure 5: Molecular structure of oxaliplatin.

Despite the difference in chemical structure, it is evident from previous experiments that Pt(II) based chemotherapeutic compounds induce nucleolar stress.[11] Thus, the goal of these experiments was to compare and contrast the compounds effects on nucleolar stress-induction by observing the reversibility of nucleolar stress when the compounds are removed from cells and replaced with drug free media. The drug free chase experiments were employed based on the hypothesis that because the small molecule inhibitors are proposed to be DNA intercalators, they could be more easily repaired in cells upon drug removal, compared to Pt(II) based compounds which formed more stable complexes with DNA that are more difficult to repair.[8] ActD and BMH-21 have also both been observed to induce reversible rRNA transcription inhibition.[45,46] However, there have been more conflicting reports for CX-5461, most indicating that it causes nucleolar stress via irreversible inhibition of rRNA transcription.[41,47]

Before conducting these comparative experiments, drug treatment concentrations for each compound needed to be determined. ActD and oxaliplatin have been frequently used in previous experiments at clinically relevant concentrations of 5 nM for ActD and 10 μM for oxaliplatin, and as such these concentrations were used for these experiments as well. For BMH-21, literature has indicated that concentrations between 1 and 5 μM were used for studies investigating rRNA transcription inhibition. Thus it was determined that the concentration for BMH-21 would be 3 μM for the reversibility experiments after completion of concentration

curve experiments.[25] The drug treatment concentration of CX-5461 was determined through the conduction of two experiments. First, concentrations between 0.05 and 5 μM have been reported in previous literature, so to determine a clinically relevant concentration of CX-5461 that would still induce nucleolar stress, treatments in a range of concentrations marginally above and below the reported IC-50 value for A549 cells were tested using NPM1 redistribution assays.[33]

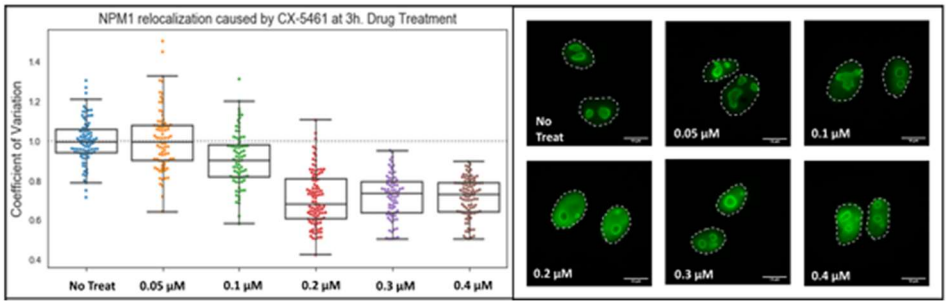

Figure 6: CX-5461 Concentration Curve

A549 cancer cells were treated with varying concentration of CX-5461 and NPM1 relocalization was quantified as a marker for nucleolar stress. A concentration of 0.2 μM was determined to be the minimum concentration required for nucleolar stress induction with CX-5461

The cells were treated for 3 hours with the varying concentrations of CX-5461 and nucleolar stress induction was measured by observing NPM1 relocalization. Based on this experiment, a concentration of 0.2 μM was determined to be the treatment concentration to be used for the reversibility experiments. The reversibility concentration was then investigated, and it was determined that at low concentrations after a 3 hr drug treatment and a 24 hr chase period in drug free medium, CX-5461 induced nucleolar stress was reversible. At drug treatments above 0.5 μM, nucleolar stress induced by CX-5461 was irreversible. This result is consistent with previous published literature indicating irreversible stress induction by CX-5461.[41,42]

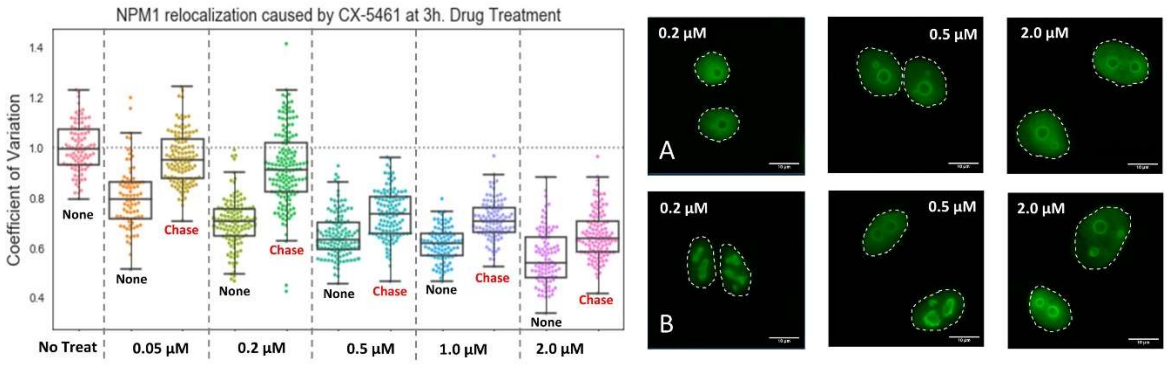

Figure 7: CX-5461 Chase Concentration determination

A549 cells were treated with varying concentrations of CX-5461 for 3 hours and then followed with a 24 hour drug-free medium incubation. 0.2 µM was the highest concentration at which nucleolar stress was reversible.

After determining experimental concentrations of the treatments of each of the small molecule compounds, the comparative experiments were conducted. The cells were treated for 3 hours with small molecule nucleolar stress-inducing compounds, followed by replacement of drug-free medium for 24 hours during the chase step. Below are the results of the experiments.

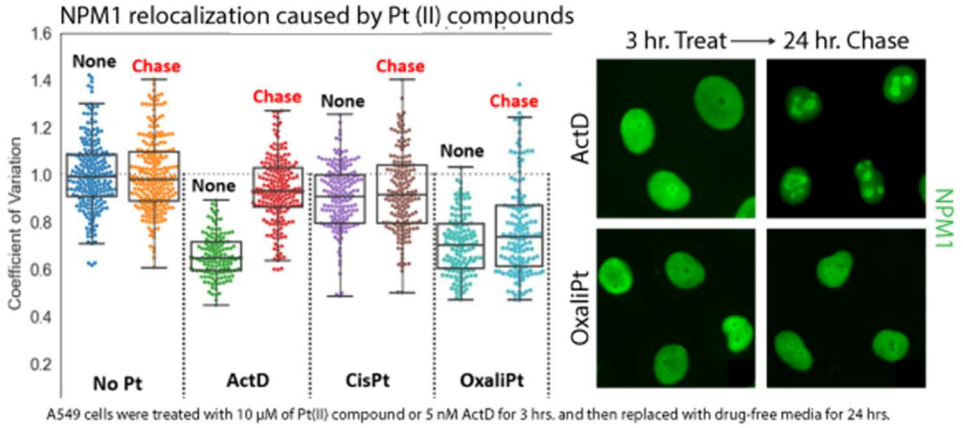

Figure 8: NPM1 relocalization of Pt(II) compounds with chase step

A549 cells were treated for 3 hours with ActD (positive control), cisplatin, and oxaliplatin followed by a 24-hour incubation with drug-free medium. Irreversible nucleolar stress was observed in cells treated with oxaliplatin.

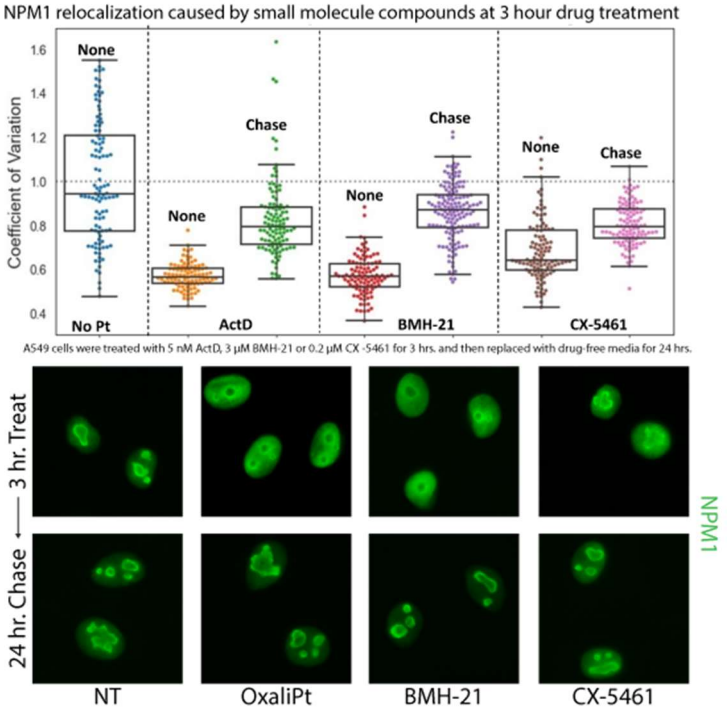

Figure 9: NMP1 relocalization of small molecule inhibitors with chase step

A549 cells were treated for 3 hours with ActD, BMH-21, and CX-5461 followed by a 24-hour incubation with drug-free medium. In contrast to the Pt(II) compounds, reversibility of nucleolar stress was observed for all 3 small molecule compounds.

It was observed that unlike the small molecule compounds, the cells treated with nucleolar stress-inducing Pt(II) compounds exhibited NPM1 relocalization that persisted even following the 24 hour chase step in drug-free medium. The CV values of the cells following the chase step for the Pt(II) compounds did not return to the no treatment control levels, while the CV values for the small molecule inhibitor compounds did return to control levels. This suggests that Pt(II) nucleolar stress-inducing compounds are potentially working through a mechanism that is distinct from the small molecule nucleolar stress-inducing compounds ActD, BMH-21, and CX-5461. This may be related to more stable interactions that are known to occur when Pt(II) compounds bind to DNA and other biomolecules. This is in contrast to the transient intercalative mechanism of the small molecule stress-inducing compounds, and may explain the reversibility of nucleolar stress that they exhibit. The comparison of Pt(II) nucleolar stress-inducing compounds to the small molecule inhibitors, suggests that Pt(II) compounds are not likely working through the same mechanisms of the small molecule stress-inducing compounds, and that the Pt(II)-induced nucleolar stress mechanism may involve irreversible binding of platinum compounds to biomolecules like RNA, DNA, or proteins.

Pt(II)-induced nucleolar stress and cell cycle regulation

Following the comparison of nucleolar stress-inducing Pt(II) compounds to small molecule nucleolar stress-inducing agents, studies were next done to help determine the role of cell cycle regulation in Pt(II)-induced nucleolar stress. The nucleolus is known to house many proteins that are involved facilitating responses to various stimuli that disrupt cellular homeostasis.[48] Depending on the severity of stress induced by the stimuli, nucleolar proteins will facilitate cell cycle arrest or activate apoptotic pathways.[48] Nucleolar proteins serve a variety of

functions and regulate cell cycle arrest and apoptosis in two primary ways. One mechanism is displacement of nucleolar proteins to other parts of the cell for signaling and activation of cell cycle arrest or apoptotic pathways. The other is the spatial isolation of negative cell cycle regulators that results in cell cycle arrest or apoptosis. Both of the two pathways involve activation of tumor suppressor protein p53, which has a wide range of roles in cell cycle arrest and apoptosis.[49,50] Due to these close connections between the nucleolus and the cell cycle, an investigation into the roles of cell cycle on nucleolar stress was warranted.

Previous works has demonstrated that cisplatin and oxaliplatin have unique effects related to the cell cycle in cancer cells.[51–53] cisplatin drug treatments results in a significant decrease in the number of cells in the G_1 phase and a corresponding increase in the number of cells in S and G_2 phases[52], indicating S and G_2 cell cycle arrest. In contrast, oxaliplatin leaves the number of cells in the G_1 phase relatively unchanged and decreases the number of cells in the S and G_2 phase[54], suggesting that oxaliplatin induces G_1 cell cycle arrest. Because of these effects observed by Pt(II) chemotherapeutic compounds on the cell cycle, experiments investigating the nucleolar stress response in relation to the cell cycle at G_1-S checkpoint were conducted. To do this, treatment conditions including caffeine, a known Chk1 inhibitor[27], were used in the trials. Chk1 is a protein important in cell cycle regulation and plays a critical role in DDR. Inhibition of Chk1 activity prevents progression of cells from G_1 phase to S phase and does not allow for DDR to occur, which primarily exists in the S phase. To investigate whether Pt(II)-induced nucleolar stress relies on Chk1 being active, experiments comparing the NPM1 redistribution of cells treated with and without caffeine for Chk1 inhibition were conducted at multiple treatment times.

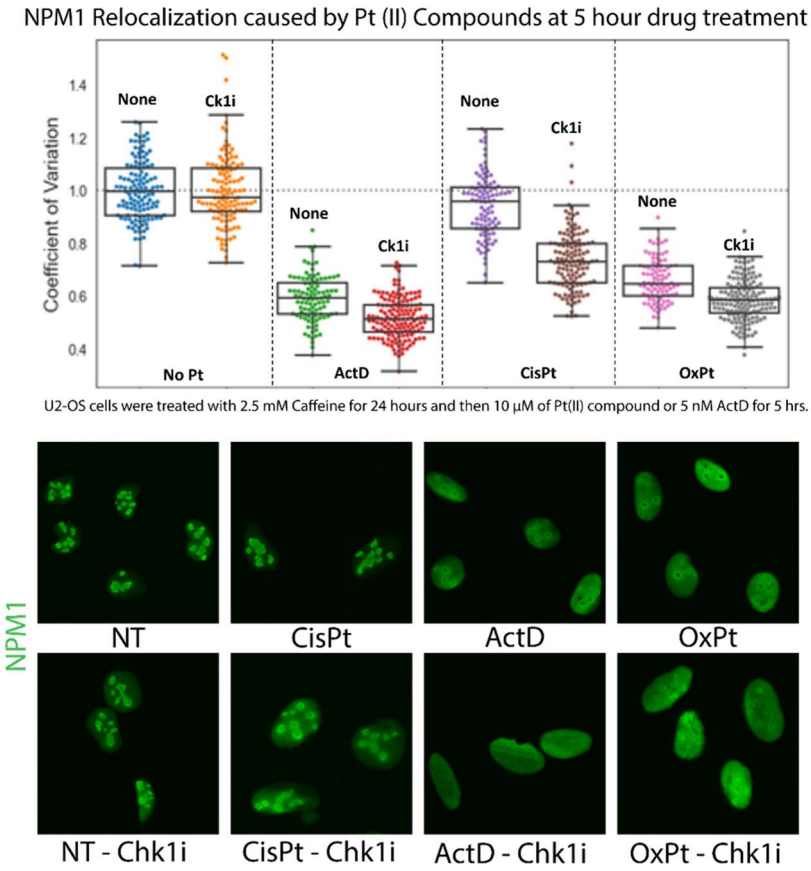

Figure 10: NPM1 relocalization with Chk1 inhibition and 5 hr treatment with Pt(II) compounds.

U2-OS cells were treated with and without caffeine for Chk1 inhibition, followed by 5 hour treatment with platinum compounds.

After 5 hours of drug treatment in the presence of Chk1 inhibitor, NPM1 relocalization is observed in cells with cisplatin, oxaliplatin, and ActD treatment. In contrast, in cells treated for 5 hours with the same compounds in the absence of Chk1 inhibitor, NPM1 relocalization is observed for oxaliplatin and ActD treatments, but no relocalization occurs with cisplatin treatment. Similar observations were observed at 24 hour drug treatment in the presence of Chk1 inhibitor, with cisplatin inducing an even more severe nucleolar stress response when Chk1 is inhibited. Furthermore, there was minimal NPM1 relocalization observed in cells treated with

Chk1 inhibitor in the absence of drug treatment, ensuring that nucleolar stress observed for cisplatin with Chk1 inhibition is not a result of the inhibitor itself.(need to include cell images for this still).

This surprising finding indicates that cisplatin, which primarily acts through DDR, is able to induce nucleolar stress when DDR is inhibited and cells are arrested in the G_1 cell cycle stage. The exact mechanism behind this observation is still not well understood but make involve Chk1 or other proteins involved in G_1 cell cycle arrest. Future studies will focus on further understanding how cisplatin causes nucleolar stress with Chk1 inhibition and investigating how this relates to the Pt(II)-induced nucleolar stress pathway and its potential divergence from the DDR pathway.

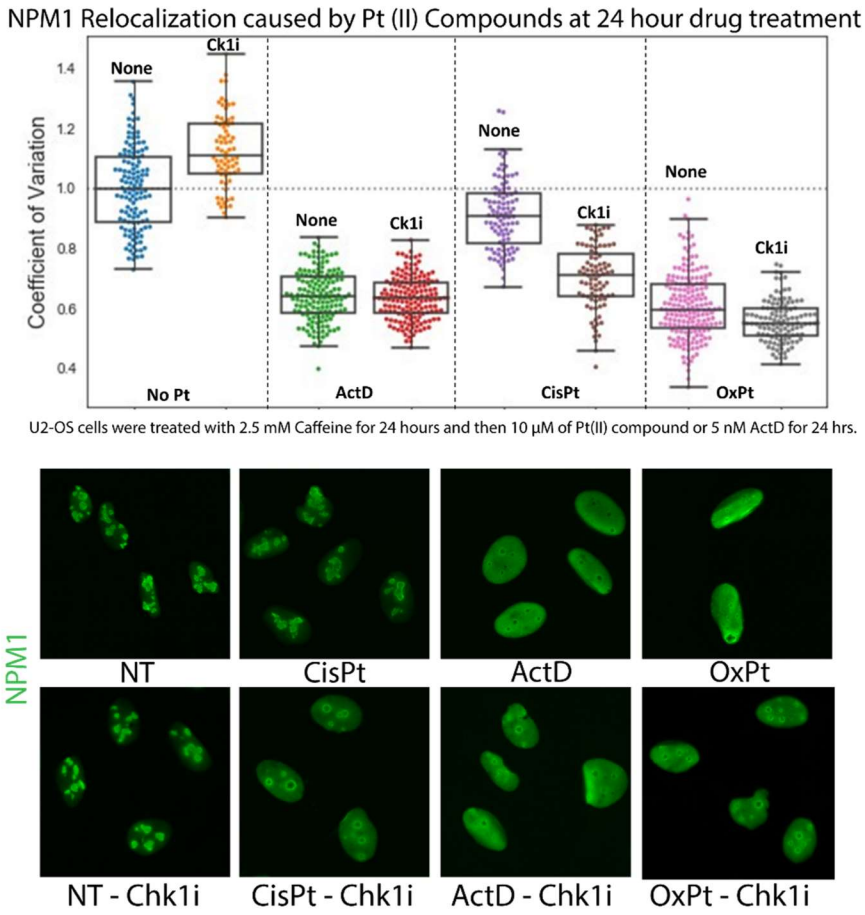

Figure 11: NPM1 relocalization after 24 hour treatment with Pt(II) compounds and Chk1 inhibition

U2-OS cells treated with drug treatments with and without caffeine in the drug treatment medium. Similar to the 5 hour trials, the experiment revealed that cisplatin induces NS when cotreated with Chk1 inhibitor caffeine.

Conclusion

The goal of this investigation was to gain insights into the mechanisms of platinum based chemotherapeutic compounds and further characterize the unique Pt(II)-induced nucleolar stress response pathway. The two main experimental designs were used in these studies, both utilizing NPM1 relocalization as an indicator of nucleolar stress induction. The first set of experiments focused on comparing nucleolar stress-inducing Pt(II) compounds to other small molecule nucleolar stress agents ActD, BMH-21 and CX5461. The second set of experiments investigating the role of the cell cycle in Pt(II)-induced nucleolar stress, specifically related to Chk1 inhibition and G_1 cell cycle arrest.

Prior to beginning the experiments comparing Pt(II) compounds to small molecule stress inducing agents, concentration gradients were used to determine relevant treatment conditions for CX-5461 and BMH-21. Surprisingly, these studies revealed that the reversibility of nucleolar stress induced by CX-5461 was concentration dependent, while no such trend was observed for BMH-21 or ActD. Figure 6 and 7 show the results of these experiments, where cells were treated with varying concentrations of CX-5461 or BMH-21 for 3 hours, followed by a 24-hour drug free chase period. From these results it was determined that an ideal experimental concentration for CX-5461 was 0.2 μM, as nucleolar stress was no longer reversible at higher concentrations. This unique concentration dependent reversibility trend for CX-5461 has not before been shown in the literature and warrants future investigations.

After determining the experimental concentrations for the small molecule drugs, experiments were conducted to compare their nucleolar stress reversibility profiles to those of stress-inducing Pt(II) compounds. Unlike small molecule stress inducing agents, Pt(II) compounds caused irreversible nucleolar stress, which persisted after drug removal. These

results suggest that there may be distinct differences between the mechanisms of the nucleolar stress inducing compounds tested. Figures 8 and 9 show the results of the reversibility trials, where cells were treated for 3 hours with Pt(II) compounds or small molecules, followed by a 24-hour incubation of drug free medium. These results demonstrate that Pt(II) induced nucleolar stress is irreversible as NPM1 remained distributed throughout the nucleus after the drugs were removed. This suggests a difference in mechanism between the Pt(II) compounds and ActD, BMH-21 and CX-5461, and may be related to the less permanent intercalation to DNA by small molecules, compared to cross-link for with Pt(II) compounds.

Studies were also done to investigate the link between the cell cycle and nucleolar stress and how it could be leveraged to gain insights into the nucleolar stress mechanisms of Pt(II) compounds. In these experiments, cells were treated with the Chk1 inhibitor, caffeine, to cause G_1 cell cycle arrest and then introduced to Pt(II) compounds and NPM1 relocalization was observed. Figures 10 and 11 show two different experiments with different drug treatment times in the presence and absence of Chk1 inhibitor. Results indicate that with Chk1 inhibition, cisplatin, which does not induced nucleolar stress under regular conditions, is able to induce nucleolar stress as early as 5 hours after drug treatment. These findings indicate that nucleolar stress may be related or dependent on the cell cycle, specifically in the G_1 phase. It could also indicate the involvement of Chk1 in the Pt(II)-induced nucleolar stress pathway, and further studies are needed to fully understand this relationship.

Although the work present here has helped to better characterize the Pt(II)-induced nucleolar stress response pathway, future works are still required to fully understand the molecular mechanisms by which these compounds induce their responses in cells. More specific investigations into the exact targets of Pt(II) based chemotherapeutic compounds is an area of

particular interest. Studies focused on Pt(II) compound interactions with specific proteins involved in apoptotic pathways are currently under investigation in the DeRose and other research laboratories. For example, recent work has shown correlations between the NF-κB pathway and nucleolar stress, and interactions between proteins involved in this pathway and Pt(II) compounds could be investigated.[55,56] Additionally, current research in the field has primarily focused on Pt(II)-DNA and Pt(II)-protein complexes, but studies has also shown that Pt(II) compounds bind to RNA and form complexes in the cell.[57,58] Investigations on Pt(II)-RNA interactions could potentially be another future area of research to further characterize the Pt(II)-induced nucleolar stress pathway.

This investigation has shown that small molecule compounds and Pt(II) chemotherapeutic compounds have different mechanisms, and that the nucleolar stress pathway induced by Pt(II) compounds is irreversible and may be caused by strong interactions with biomolecules. In addition, it was found that nucleolar stress induced by Pt(II) compounds is tied to the cell cycle and that cell cycle arrest could redirect cell death pathways to nucleolar stress rather than DDR.

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