Apoptotic Properties of Platinum Antitumor Agents Phosphaplatins

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A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

By

Homa Dezvareh

May 2016

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ABSTRACT

Phosphaplatins are a group of non-DNA binding platinum compounds that exhibit excellent *in vivo* and *in vitro* efficacies against a variety of cisplatin- and carboplatin-sensitive and resistant ovarian cancers. Although, combinations of cis- or carbo-platin and paclitaxel therapies continue to be the choice of treatment for ovarian cancer, many patients develop resistance to the treatment. Phosphaplatins are pyrophosphate bound platinum-(II) and (IV)-platinum complexes that are bi-negatively charged at or near neutral pH. Both Platinum (II) and platinum (IV) complexes do not induce the overexpress of any DNA repair genes, consistent with the no-DNA binding property of phosphaplatins. In this report, we show the apoptotic properties of two representative compounds, (R, R-1,2-cyclohexanediamine)-(dihydrogen pyrophosphato)platinum (II) (RRD2) and (R,R-1,2-cyclohexanediamine)-trans-dihydroxo(dihydrogen pyrophosphato)platinum (IV) (RRD4) through a variety of experiments as described below.

Phosphaplatins are found to activate and upregulate death receptors on cell surface such as *FAS, DR5,* and TNFR1. These three death receptors follow the common signaling path to trigger apoptosis by an extrinsic pathway via Death Inducing Signaling Complex (DISC) by recruiting FADD and procaspase-8 and activating caspase-8. A direct binding of FAS by platinum is implicated as the platinum compound was found to be co-localized in the lipid rafts.

The PTEN-PI3K pathway involvement was confirmed by the down regulation of both

PI3K and p-AKT. Phosphoplatins also upregulate tumor suppression genes P53 and PTEN. Apoptosis by p53 signaling follows intrinsic pathways involving BCL-proteins. In particular upregulation of BAX, PUMA, and downregulation of BCL2 were observed. To further confirm p53-signailng, we have identified a number of *p53* target genes that include AEN, CYFIP2, TP53I3, TP53INP1, DPYSL4, LRDD, DRAM1, and a few others.

Our data shows that phosphaplatins have the potential to not only treat resistant but also advanced metastatic ovarian cancers.

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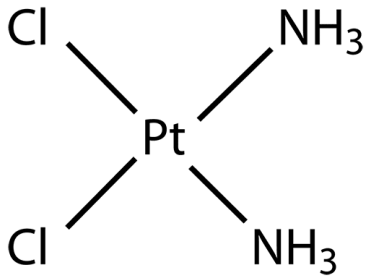
1 INTRODUCTION

1.1 Phosphaplatins

Cancer is one of the leading causes of morbidity and mortality worldwide; one in eight deaths is cancer-related. During the past two decades, there has been a remarkable progress in inventing and designing new drugs to treat variety of cancers. These cancer-treatment agents are designed to prolong the life span and improve the quality of life of cancer patients. Cancer chemotherapy is one of the most important and aggressive treatment options available to patients. There are a number of platinum metal-based anticancer drugs such as cisplatin, carboplatin, and oxaliplatin that are widely used for the treatment of variety of cancers. These anticancer drugs have provided hope for treatment of cancer that once thought untreatable. Most of the platinum drugs covalently bond to DNA through both inter- and intra- strand binding modes and this inhibits gene transcription and DNA replication. There are two major issues that have not been resolved when dealing with cancer therapy. One of them is resistance to treatment and the second problem is toxicities due to these treatments.

Figure 1: Cisplatin (cis-diammine-dichloro-platinum)



Cisplatin is one of the drugs commonly used to treat ovarian, testicular and head and neck cancers since 1978(26). Over the years, cisplatin is known to be one of the most potent anticancer agents due to its remarkable clinical activity. It has also been very effective in increasing survival rates in ovarian cancer patients and patients with various solid tumors. However, cisplatin has its own drawbacks as a cancer therapeutic drug. Cisplatin has a high level of toxicity and in some cases tumor cells quickly develop resistance to cisplatin treatment. Also, the primary target of active cisplatin is DNA and it bonds and forms crosslinks between the two strands of the DNA. Cisplatin generally mediates cell death via P53 tumor suppressor protein and its down stream mitochondrial proteins including Bax, Bak and PUMA, Fas death receptor and its downstream signaling proteins. Therefore, apoptosis and cell cycle arrest is why ovarian cancer responds well to cisplatin-based treatments initially. But because cisplatin binding to DNA is sensed as DNA damage, it causes the activation of DNA damage repair and rescue pathways. This deactivats cisplatin and triggers a detoxification mechanism (26). Together, these series of events cause resistance to cisplatin by cancer cell lines and shortens the treatment period and results in high toxicities.

Researchers have been trying to explore new approaches and optimize new treatment options to overcome these drawbacks over the years. Phosphaplatins, invented by Dr. Rathindra Bose, is a new class of platinum-based compounds that is currently being tested in human clinical trials(14) . Our focus was initially on ovarian cancer and over time we tested these compounds on a variety of other cancer types including head and neck, lung, prostate and breast cancer.

Phosphaplatins are non-DNA binding platinum compounds that exhibit excellent

in vivo and *in vitro* efficacies against a variety of cisplatin- and carboplatin-sensitive and resistant ovarian cancers. Although, combinations of cis- or carbo-platin and paclitaxel therapies continue to be the choice of treatment for many ovarian cancers, many patients develop resistance to the treatment. Phosphaplatins are pyrophosphate bound platinum-(II) and (IV)-platinum complexes that are bi-negatively charged at or near neutral pH. Both Platinum (II) and platinum (IV) complexes do not induce overexpression of any DNA repair genes consistent with the no-DNA binding property of phosphaplatins. We have tested the apoptotic properties of two representative compounds, (R, R-1, 2-cyclohexanediamine)-(dihydrogen pyrophosphato) platinum (II) (RRD2) and (R, R-1,2-cyclohexanediamine)-trans-dihydroxo(dihydrogen pyrophosphato)platinum (IV) (RRD4) through a variety of experiments in the following chapters. Phosplatins have shown to be not only less toxic compared to carboplatin and cisplatin but also is biologically active. These agents have a very slow rate of hydrolysis and have high potential to remain under aqueous conditions for longer period of times. Unlike other commercially available drugs, phosphaplatins showed no effect to temperature fluctuations and high stability at neutral pH for extended periods of time. In comparison to cisplatin, which loses activity after 5 hours of reconstitution, phosphapltins showed no change in their activity after a month of storage.

One of the main questions about phosphoplatins is on their mechanism of action. In our lab we have been trying to study how these compounds work to treat cancer, and which pathway are involved in inducing cell death in tumor cells.

Additionally, we have significantly improved the procedure of phosphoplatin synthesis.

Initially synthesizing phosphoplatins required large reaction volumes, which only produced small quantities of products. Part of the reason is the low solubility of the starting platinum reactants. We have invented a method for synthesizing these compounds on a large scale, that eliminates the need to use large volumes of starting materials by increasing the solubility of those starting materials in a low volume reaction mixture. This new process significantly reduces the reaction time. This new process also makes it easier to recycle the unreacted platinum complex and pyrophosphate after the first batch of phosphapltins is synthesized. We can simply filter the waste products from the reaction mixture and add appropriate starting materials to yield another batch of phophaplatins.

1.2 Pigment epithelial derived factor (PEDF)

One of the main issues with chemotherapy is its toxicity. Studies have shown that most platinum-based anticancer drugs that are currently available in the market including cisplatin, carboplatin and oxaliplatin can cause severe toxicities including nephro-, neuro-, and oto-toxicities. For example, oxaliplatin showed excellent anticancer properties but it has only been approved to treat colon cancer due to its severe neuro-toxicity. These neurological damages can lead to variety of diseases. As shown in the following chapters, our data show that phophaplatin can actually provide neuroprotection by inducing secretion of Pigment epithelial derived factor (PEDF)

PEDF is a glycoprotein that is mainly responsible for protecting neurons and is produced by retinal pigment epithelial cells. It was originally discovered in cultured fetal retinal pigment epithelial (RPE) cells (99). PEDF originally was detected in brain and blood at high concentrations within the retina and in the Vitreous (100).

Research has shown that PEDF mRNA is found in most tissues. It is also abundantly expressed in many human organs, normal cell types (neuronal and non-neuronal) and tumors (5).

Studies have concluded that against various exogenous and endogenous insults, PEDF exhibited a remarkable ability to provide tremendous protection of neurons. This protein has shown to prevent ischemia-induced retinopathy (101), diabetic retinopathy**,** and other neurological disorders (102).

Furthermore, PEDF has been shown to have a neuro-protective property, which is from

inhibition of abnormal blood vessel growth of ocular neovascularization (5) and this is independent of its role in the inhibition of angiogenesis (5).

Furthermore, Studies have shown that in metastatic cancer patients, PEDF is either decreased or deleted. This gradual loss of PEDF is associated with poor prognoses of variety of metastatic cancers including colorectal, breast, lung, ovarian, and others.

PEDF is also known as a regulatory factor for various pathways including lipid metabolism (103) and stem cell renewal (104) in addition to its neurotropic and neuroprotective roles.

Researchers have targeted PEDF as a gene therapy to treat a variety of diseases including macular degeneration (105) and neuro-degeneration (102) and cancer metastases because of its neuroprotective and antiangiogenic properties. Unfortunately, such strategy has met with limited success due to the inefficient delivery and/or instability of PEDF.

In our initial testing of phosphaplatins, we came across the overexpression of PEDF in cells treated with phophaplatin compouds. We have since performed a series of experiments to assess and confirm PEDF up regulation and the results are presented in the following chapters.

1.3 Apoptosis

Initial studies on phosphaplatins showed that Fas (CD95/APO-1) death receptor is upregulated among several other genes such as Bax, Puma and p53.

Fas belongs to type I family of transmembrane death receptors and is activated by ligand binding to its extracellular domain. Research shows that for caspase 8 to become activated, ligation of death receptors such as Fas by its ligand is needed. These events lead to Fas receptor trimerization, recruitment of FADD (Fas Associated Death Domain) to the cytoplasmic side of the receptor and formation of Death Inducing Signaling Complex (DISC) at the interacellular tail of Fas. Next, other down stream signallings are activated to induce apoptosis.

We focused on both extrinsic and intrinsic cell death because mithochondrial cell death pathway can also be activated by receptors like Fas. In order to confirm the intrinsic cell death pathway involvement further studies have been performed and the results are presented in Chapter 3. Research in the field of apoptosis has shown that there is a considerable cross talk between various cell-signaling pathways leading to cell death (48).

Our initial studies on phosphaplatins were mainly focused on P53 tumor suppressor genes. P53 has a remarkable role in cancer suppression by preventing aberrant cell growth due to its role in inducing apoptotic cell death. P53 induced apoptosis is achieved by either stimulating the death receptor pathway with caspases or release of toxic proteins from the mitochondria. These two pathways can be stimulated and combined by p53; this

can be achieved by activating the common downstream BID gene to induce apoptosis. To further assess the involvement of P53 in inducing apoptosis caused by phsophaplatins, we have performed gene expression studies in the results section (mention which chapter).

1.4 Cell Survival

PI3K/AKT pathway has a significant role in key processes such as cell growth, metabolism, survival, motility and proliferation, by relaying mitogenic signals to intracellular effector molecules (106). It is one of the most frequently altered signaling pathways during the development and progression of cancer (107). Phosphoinositide 3-kinase (PI-3 kinase or PI3K) family are important lipid kinase signaling factors. PI3K pathway is constitutively active in up to 50% of human tumors. PI3K pathway in solid tumors such as ovarian cancers has been extensively investigatd (108).

Akt is the down stream mediator of PI3 Kinase pathway and is located upstream of various important signaling pathways within the cell. By phosphorylating intracellular substrates which affect crucial cellular processes such as growth and survival, Akt amplifies and transmits survival signals. However, by phosphorylating, Akt inactivates downstream substrates such as BAD, p21 and release of cytochrome c, which are important apoptosis executors. Under conditions of stress signaling, The PI3K/Akt pathway which negatively regulates proapoptotic proteins such as p53via BAD and BAX, is counteracted by the activation of PTEN gene and the down regulation of Akt by dephosphorylation.

PTEN also acts as a tumor suppressor gene, and can activate p53 directly or indirectly.

Research has shown that loss of PTEN causes a decrease in p53 expression levels. As

such, we have assessed how these genes and their protein products are affected during phosphaplatin cancer treatments.

1.5 significance of this study

In order to further study the mechanism of action of RRD2 and RRD4, we will be using a combination of *in vitro* and *in vivo* methods to assess how these compounds function in comparison to other drugs.

To demonstrate the properties of our compounds against resistant human ovarian cancers, we have selected a cisplatin-sensitive (A2780) cell line, a cisplatin- and carboplatin-resistant (C30) cell line. In this study we are focusing on different signaling pathways to dissect the mechanism of action of phosphaplatins.

2 Materials and Methods

2.1 Platinum Compounds

Low volume synthesis of RRD2 and RRD4

Phosphaplatins were synthesized in our lab and originally they were invented by Dr. Rathinddra Bose. We have suggested a process for recycling the used unreacted material at the end of the synthesis and also a low volume synthesis. In the low volume synthesis, platinum reagents of general formula PtA2X2 are added to a saturated solution of HnNa4-nP2O7 which is dissolved in 25 ml of distilled water at a pH of preferably about 8, and at a temperature of 60°C. Next, the reaction is stirred for approximately 9-12 hours. After stirring, the volume of the reaction needs to be decreased to 5-7 ml using a rotary evaporator at 48 °C. Once the reaction mixture is at 5 ml, the pH is adjusted with concentrated HNO3 to about 1.5-2. The reaction mixture is left on ice for 5 minutes to precipitate out the phosphaplatins, and then the phosphaplatins are filtered out. We then leave the precipitated RRD2 compound in a desiccator to dry for one or two days. (4)

To make RRD4, after 9-12 hours of stirring at 60°C, we add 1 ml of hydrogen peroxide (1.0 mL; 30%) followed by another 1-hr incubation at 60 °C. Then we decrease the volume to a total of 5-7 ml and follow the process as described above.

Next the temperature of the filtrate is adjusted to about 40° C. and then let the reaction stand for about two hours, to precipitate out dimers and oligomers, if any. Finally, the pH of the filtrate is adjusted to between 6.0 and 8.5, preferably pH 8. Afterwards, appropriate amounts of pyrophosphate and starting platinum reagents are added to meet

starting conditions, and the above steps are then repeated to yield further phosphaplatins at 60° C. The recycling process is the same for both RRD2 and RRD4 except the addition of hydrogen peroxide at the end of the synthesis. (4)

2.2 Immuno-Fluorescence

A 6-well plate containing pretreated cover slips was seeded with 500-1000 A2780 human ovarian cancer cells in 2.5 ml of RPMI 1640 media (Lonza Walkersville, Frederick, Md.) containing 2.0 mM L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah), and 0.25 units/mL recombinant human insulin (Sigma Aldrich, St. Louis, Mo.), 100 U/ml penicillin-streptomycin (Lonza Walkersville, Frederick, Md.). Once cells reached 80% confluency, they were treated with phosphaplatin compounds, RRD2 and RRD4 at various time intervals (3, 6, 12, or 24 hours) at various concentrations. Each of the cover slip was washed with PBS three times, and the cells were fixed with freshly prepared 4% paraformaldehyde at room temperature for 20 minutes. The fixed cells were then washed with PBS for three times. These cells were then permeablized and blocked with 7.5% bovine serum albumin, BSA/PBS/0.01% triton-X100 at 37° C. for 60 minutes followed by washing the cells with PBS twice. The cover slips were treated an anti-PEDF mouse primary antibody (Cell Signalling Technology Inc., Danvers, Mass.) diluted 1:300 in 7.5% BSA/PBS/0.01% triton for 12-15 hours 37° C. and washed with PBS for three times. The coverslips were incubated with secondary Alexa Fluor 488-conjugated anti-mouse IgG (FITC-Green) (Invitrogen, Carlsbad, Calif.) at a 1:5000 dilution in 7.5% BSA/PBS/0.01% triton-X100 for 1 hour at 37° C. in the dark. The cover-slips were then washed with PBS for another three times. In addition, some treatments were stained for beta-Actin and was

stained with Phalloidin (Rhodamine) (Red) (Invitrogen, Carlsbad, Calif.) (7 μl/ml) in 7.5% BSA/PBS/0.01% triton-X100 for 1 hour at 37° C. in the dark. The moist cover-slips were then mounted onto a microscope slide with Ultracruz mounting media containing DAPI (4′,6-diamidino-2-phenylindole) (Blue) (Santa Cruz Biotechnology, Santa Cruz, Calif.) (for identifying the nucleus) and images were then collected on an Olympus FV-1000 inverted stage confocal microscope using a Plan Apo N 60×1.42NA oil immersion objective and processed by FluoView software (5).

2.3 Protein Expression by Western Blot

Human ovarian cancer cells (approximately 1×10^6 cells in 60 mm3 plates at 80-90% confluence) were treated with 20 μM RRD2 and 25 μM RRD4 for 3, 6 and 12 hrs. After washing with PBS for three times, total protein was extracted by treating the washed cells directly with 200 μl of RIPA (Sigma, St. Louis, Mo.) or MPER (Pierce, USA). Protein extract samples (50 μg) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were washed with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween-20], blocked with 5% skim milk and 1% BSA overnight at 4 ^{0}C, and incubated with the mouse anti-PEDF (Santa Cruz Biotechnology, USA) primary antibody at 1:500 dilutions in TBST with 5% skim milk and 1% BSA overnight at 4° C. The membrane was then washed with TBST, and then incubated with the secondary antibody, which is goat anti-mouse IgG conjugated to horseradish peroxidase at 1:2500 dilution for 1.5 hours at room temperature. The blots were developed with enhanced chemiluminescence (ECL) (Pierce, USA) and exposed to X-ray film. The membranes were stripped with stripping solution (for 1.5 hrs and re-

probed with mouse anti-β-actin (Santa Cruz Biotechnology, USA) primary antibody for determining loading. Essentially, PEDF protein expression was upregulated following 3 hrs, 6 hrs, and 12 hrs treatment with 20 μM and 25 μM of compound I and IV in human ovarian cancer cells (A2780)(5).

2.4 Clonogenic Assay

Human ovarian cancer cells, A2780, A2780/C30 (cross resistant to 30 lM cisplatin and 100 lM carboplatin), and OVCAR-5 and -10, were obtained from Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured on monolayer using RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and 0.25 units/ml insulin and penicillin/streptomycin (100 units/mL) in an incubator (37 °C) continuously gassed with 5% CO_2. Cells were maintained in the exponential growth phase. The IC_{50} values were determined using a clonogenic assay. Briefly, 500–700 cells from a single cell suspension were plated onto 60-mm petri plates for 24 h before treating with platinum compounds to permit cell attachment. These cells were then treated with platinum compounds (50 nM to 75 nM) for 1-24 hr, the platinum compound-containing media were decanted and replaced with a fresh medium. Triplicate plates were set up for each concentration. These plates were returned to the 37 °C incubator for 7 days for colony formation. Colonies were fixed and stained using 2% crystal violet in 4% formaldehyde. Colonies containing more than 50 cells were scored. The number of scored colonies from the triplicate plates was averaged, and this number was divided by the number of cells plated to obtain a value for the fraction of cells forming colonies. These values for fraction of cells forming colonies were then corrected for plating efficiency by dividing them with the number of cells forming colonies in plates

that were not treated with Pt compounds(48).

2.5 Calculation of IC_{50} values

The cell survival curves, fractions of cells capable of forming colonies vs. drug concentrations, displayed an exponential decay. Since these curves displayed an exponential decay of cell survival as a function of concentration of platinum concentration, these data were fitted to an equation:

$$(NC) = (NC)_0 e^{kC}$$

$$IC_{50} = 0.693/k$$

Where $(NC)_0$ and (NC) are the percentages of surviving cells at zero and various concentrations, k is the characteristic decay constant, and C is the concentration of the antitumor agents. (48)

2.6 In vivo experiments in mice and rats

Five-week old female CD1 mice (Taconic Farms Inc.) were used for maximum tolerated dose (MTD) and blood chemistry, and 28– 30-day old female Sprague–Dawley rats for plasma clearance studies. Typically, each group consists of five to ten mice or 3 rats. Upon arrival, the animals were acclimated to their surroundings for 7 days. They were handled according to the experimental protocols approved by the Institutional Animal Care and Use Committee (IACUC) at University of Houston and/or Ohio University where some of the work were performeds. A laminar flow work station with a bio- hazard hood was used to accommodate the use of platinum-based compounds and cancer

cells (48).

2.7 Maximum Tolerated Dose (MTD) Determination

To determine the maximum tolerated doses of phosphaplatins compared to placebo (PBS and 25 mM Bicarbonate solution, pH 7.4) and cisplatin (10 mg/kg), RRD2 and RRD4 (10, 30, 60 and 100 mg/kg of each drug) were administrated via intravenous injections through the tail vein. Three doses (N = 5, each group) were administered every other day over five days (day 1, day 3 and day 5). Clinical and physiological observations (weight and food consumption) were recorded on a daily basis for 31 days. On the 31st day animals were euthanized by anesthetizing with avertin solution, 0.1 mL/5 g injected intraperitoneally using a 26 gauge needle and a tuberculin syringe, and then exsanguination under anesthesia. At the end of the study the liver, lung, ovary, heart, spleen, and blood were excised, snap frozen with liquid nitrogen, and kept at 80 °C until the day of analysis(48).

2.8 *In vivo* efficacy measurements

In vivo efficacy experiments were performed using NIH SCID mice. The detailed experimental protocols for tumor growth and measurements are shown elsewhere [18]. Typically, actively growing viable cells were used to inoculate the female NIH SCID mice. Viable A2780 cells were suspended in pre-warmed PBS and were injected (1–2 10^6 cells) subcutaneously in the flank of each animal. The animals were randomly divided into control and treatment groups after the tumor had reached a palpable size (150–200 mm^3). The therapeutic compounds were administered

intraperitoneally once the tumors reached a size of >100 mm^3. Tumors were measured using digital calipers throughout the study (48).

2.9 Clearance of platinum compounds in plasma

To examine platinum accumulations in and clearance from plasma, RRD2 (10 mg/kg in PBS and 25 mM Bicarbonate solution pH7.4) was administered via the tail vein injections in 26 – 28 days old female Sprague–Dawley rats (Charles River, Spencerville, OH) (N = 3, per group) after one week of acclimation. Animals were euthanized per protocol as indicated above at 10 min, 30 min, 1, 3, 6, 9, 12, 18 and 24 h. The freshly collected blood was immediately transferred to an EDTA coated centrifuge tube and vortexed gently to avoid coagulation and analyzed with Hemavet 950 (Drew Scientific, Dallas, TX). Plasma (500 lL) was collected from the remaining fresh blood and laced in an Amicon Ultra filter device with MWCO of 3 K and centrifuged 14 000g for 30 min at room temperature. The filtrate and concentrate was collected and stored at 80 °C prior to GFAAS analysis (48).

2.10 Blood chemistry

Blood components such as white blood cells, neutrophils, and platelets among other constituents were monitored for seven days after a single injection of placebo (PBS and 25 mM Bicarbonate solution pH7.4), RRD2 (10 mg/kg), RRD4 (10 mg/kg), carbolplatin (60 mg/kg), or cisplatin (7 mg/kg) by i.p to CD1 mice (N = 10, each group). Blood samples (100 μL) of peripheral blood were collected from cardiac puncture into EDTA-anti-coagulated coated centrifuge tubes (N = 5–10). The freshly collected blood was vortexed gently to avoid coagulation. Samples of 25 μL of blood were measured for total blood counts on Veterinary Multi-species Hematology System, Hemavet 950 (Drew Scientific, Dallas, TX)(48).

2.11 Platinum content measurements in blood and tissue samples

Platinum contents in urine, blood, and serum or plasma were determined by digesting 30–50 μL of the biological sample in 500 μL of HNO_3 for 15 min at room temperature. An antifoaming agent (200 lL; 0.6% Triton X-100 and 0.6% isopropanol) was added to the sample, and it was heated for 1 h at 70 °C. The solution was oxidized by adding 50 lL of 30% H_2O_2 for another hour at 70 °C or overnight at room temperature covered with aluminum foil to avoid evaporation. The digested sample was brought up to 1000 lL by adding 0.1 M HCl, of which an aliquot of 20 μL was analyzed. Frozen excised organ tissues (kidney or liver) were thawed at room temperature and dried on a filter paper. A small sample (75 mg) was transferred to a Pyrex test tube and sheared with scissors to very fine particles. The sheared samples were treated with 500 lL of

HNO_3, and a mechanical homogenizer was used to make a complete homogenized solution. The sample was left at room temperature for 15min. The antifoaming agent and oxidizing agent were then added as mentioned above, and the sample was brought up to 1000 lL by adding 0.1 M HCl. An aliquot of 20 lL of the sample was analyzed by atomic absorption (48).

2.12 Atomic absorption spectroscopy

Platinum contents were estimated on a graphite furnace atomic absorption instrument (Perkin Elmer AA-600). The instrument was calibrated using platinum standard (Perkin Elmer, Waltham, MA) in 0.1% HNO_3 or similar sample matrix(48).

2.13 Chip microarray

Cells were treated with compound at different concentration for 12 or 24 hours. The total RNA from the treated human cells were extracted by Trizol (Invitrogen) and purified by Rneasy Mini kit (Qiagen, Valencia, CA). The concentration and integrity of all RNA samples were assessed using the NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies,Wilmington, DE) and the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). 1ug of total RNA from each sample were subjected to whole-genome gene expression analysis at the Microarray Core Facility of University of Texas Southwestern Medical Center (https://microarray.swmed.edu) using the HumanHT-12 v4.0 BeadChip (Illumina,

San Diego, CA) according to the manufacturer's instructions. Microarray data were extracted using BeadStudio v3.1 software, background-subtracted, and normalized using a cubic spline algorithm. Genes differentially expressed between groups were identified using the Illumina custom error model implemented in BeadStudio. Genes were considered significantly differentially expressed when P values were less than 0.05 and the change was greater than 1.5-fold. The pathways and interaction networks that the genes involved were further analyzed by Ingenuity Pathway analysis software (www.ingenuity.com)

3 Results

3.1 Establishment of an efficient processes for large-scale preparation of phosphaplatins antitumor agents

To significantly improve the synthesis of all phosphaplatin compounds, we have invented an efficient process for synthesizing these compounds in large quantities and a process for recycling un-reacted materials from the first phosphaplatins synthesis. Increasing the concentration of reactants and adjusting the volumes accordingly can scale this efficient process up.

Another advantage of this process is its potential to recycle the mother liquor after collecting the first batch of phosphaplatins, which contains unused starting materials that are platinum complex and pyrophosphate along with the product that was not precipitated due to its inherent solubility. In this case, additional pyrophosphate is added at a concentration necessary to exceed the amount of platinum reactant for the synthesis via a kinetic controlled process. Our experiments showed that up to 25% of the starting sodium pyrophosphate ligand is consumed in the reaction, 75% of the un-reacted starting pyrophosphate can be reused in the recycle process. The first reaction is performed using ten-fold excess of pyrophosphate ligand. In the subsequent cycle, only the starting platinum complex needs to be replenished until the mole ratio of platinum:pyrophosphate reaches 1:4. At that point, additional pyrophosphate is added to meet the kinetic criteria and to avoid the formation of any dimeric products.

In a low-volume synthesis of phosphaplatins, we can create a highly soluble di-aqua-platinum(II) compound by removing the chloride or iodide ligand from the starting platinum complex, and reacting that compound with the pyrophosphate moiety. Under these conditions, the reaction yields several minor products in addition to the major monomeric phosphaplatin complexes.

Platinum (IV) complexes were also synthesized following the low-volume process by oxidizing the platinum (II) complexes with hydrogen peroxide at the end of the incubation time at pH 6-8 (4). Therefore, the invention is applicable to low-volume synthesis of both platinum (II) and platinum (IV) complexes.

The phosphaplatins are shown by a general formula (I) depicted in Figure 2, where R1 and R2 are amine ligands, and R3 and R4 are either amine or other monodentate ligands. There are also the many chemical variations of these phosphaplatin structures, as shown in Figure 2 that include *cis*-, *trans*-, racemic and enantio-pure forms where the amine ligands contain chiral centers. For the platinum(II) complexes, examples of *cis*-, *trans*- and optical isomers of general formula (I) are shown in Figure 3 with formulas (III) through (V) for, and formulas (VI) through (VIII) are for platinum(IV) complexes using a chiral amine ligand, 1,2-diamine cyclohexane. (4)

Figure 2: The general formula (I) of phosphaplatin antitumor agents where R1 and R2 are amine ligands, and R3 and R4 are either amine or other monodentate ligands.

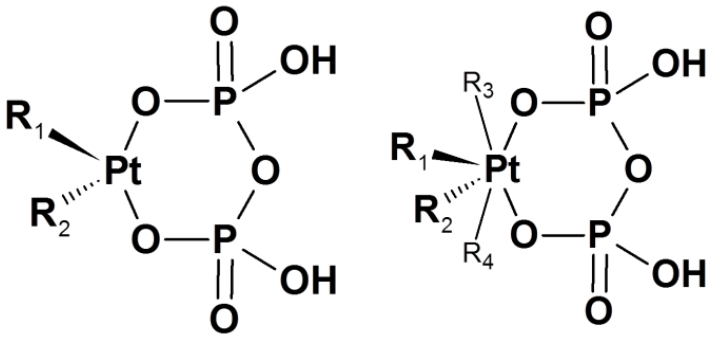
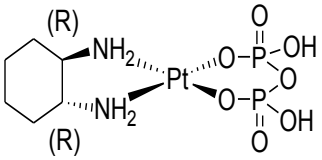
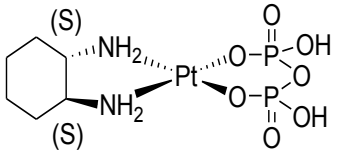


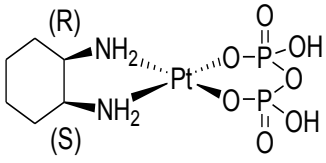
Figure 3: Shows examples of *cis*-, *trans*- and optical isomers of general formula (I) as exemplified in formulas (III) through (V) for platinum (II) complexes and formulas (VI) through (VIII) for platinum(IV) complexes using a chiral amine ligand, 1,2-diamine cyclohexane



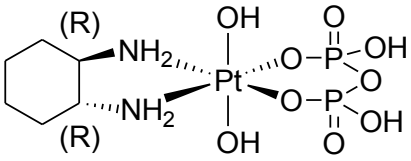
(III)



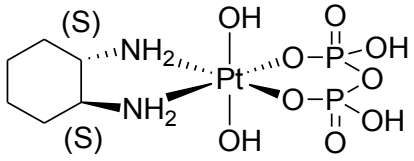
(IV)



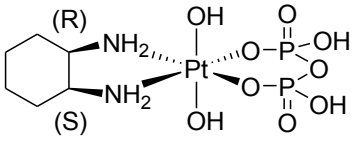
(V)



(VI)



(VII)



(VIII)

In the low volume synthesis of Phosphaplatins at 60 ^{O}C**,** we have collected samples at different time points during the process to examine and determine the quality and purity of the compound. Figure 3 shows one example of Phosphorus-31 NMR spectra of the reaction mixture recorded after 6 and 9 hours of reaction at 60 ^{O}C, and the final product isolated from the reaction. The downfield peak at 1.93 ppm is for the monomeric pyrophosphate complex used in starting material, and the peak at -5.62 is for the excess pyrophosphate ligand which is unreacted. As seen in Figure 4, there is no change in relative intensity of the product peak after 6 hr of reaction time, indicating that the reaction time can be shortened considerably due to the temperature increase from 40 ^{O}C to 60 ^{O}C. In fact, prolonged reaction times beyond 9 hr at 60 ^{O}C seemed to reduce the final product peak. Furthermore, it is possible to reduce the reaction time by increasing the temperature below the decomposition of the pyrophosphate ligand. Following the incubation period, the solution was filtered to remove any un-reacted starting material and was concentrated to 5-7 mL by rotary evaporation under vacuum at 48 ^{O}C. The pH was lowered to approximately 1.5-2 by the addition of 1 N HNO_3, and temperature was lowered to 4°C to precipitate out the product as a light-yellow powder. Cooling on ice for 5 minutes completed precipitation, and the product was isolated by vacuum filtration and washed with ice-cold water and acetone (three times at 10 mL per wash). The final product was dried under vacuum in a desiccator overnight.(4) Yields of $[Pt(C_6H_{14}N_2)(H_2P_2O_7)]$ were in the range of 0.06 g. The average yield was 50%. The volume of the starting reaction mixture can be lowered further to 7-10 mL to eliminate the concentration step. However, by doing so the yield of the final product is significantly reduced. The product obtained was fully characterized by Pt

elemental analysis, P-31 NMR spectroscopy, HPLC, and mass spectrometry. The reaction was repeated under identical conditions by lowering the temperature at 40 ^{0}C. The same product featuring identical analytical characteristics stated above was performed. The only difference is that the reaction time was extended to 18-24 hr due to the lower temperature (4).

Figure 4: Shows a Phosphorous-31 NMR spectra of a reaction mixture at 6 and 9 hours and final product.

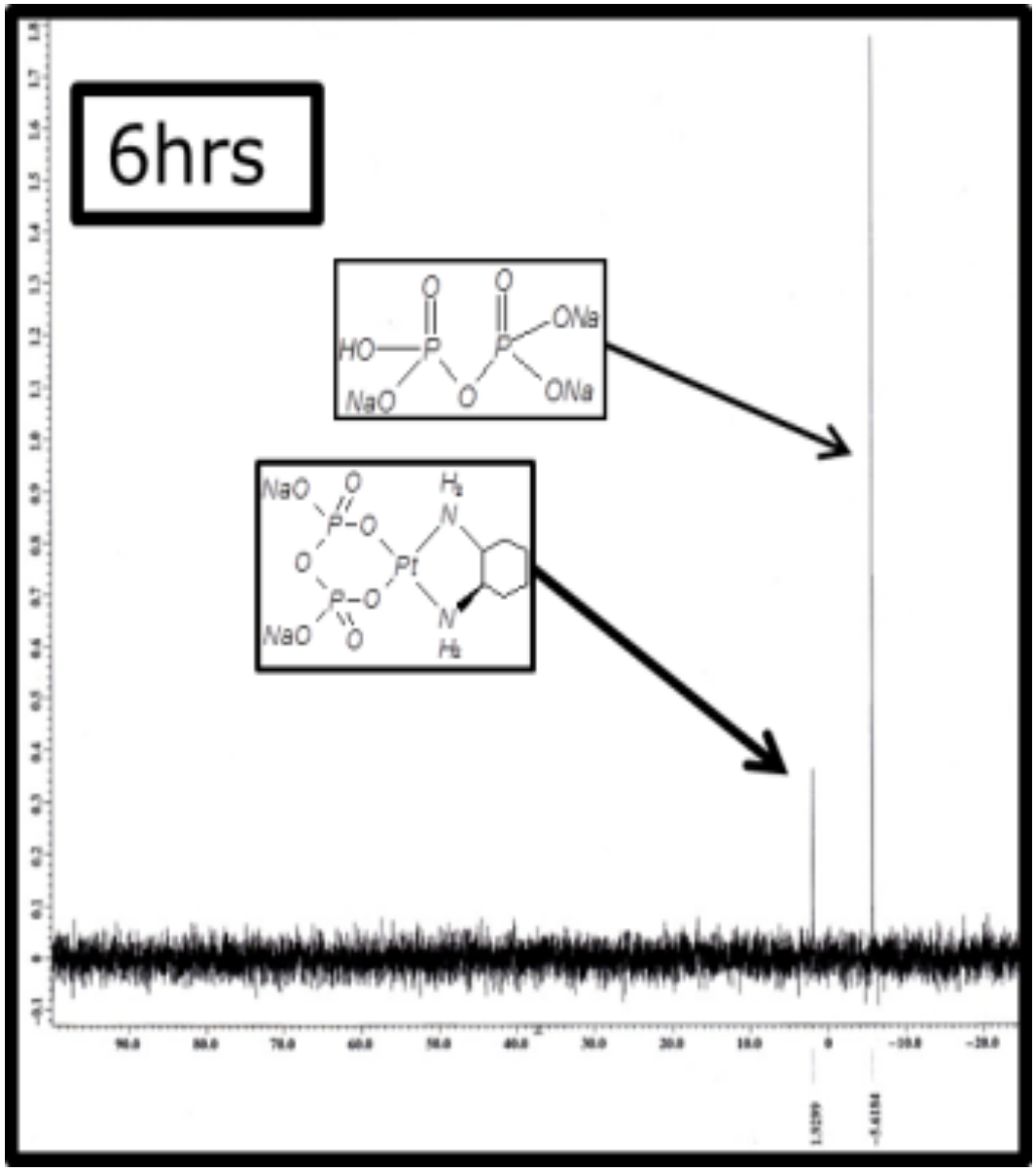


Figure 4:

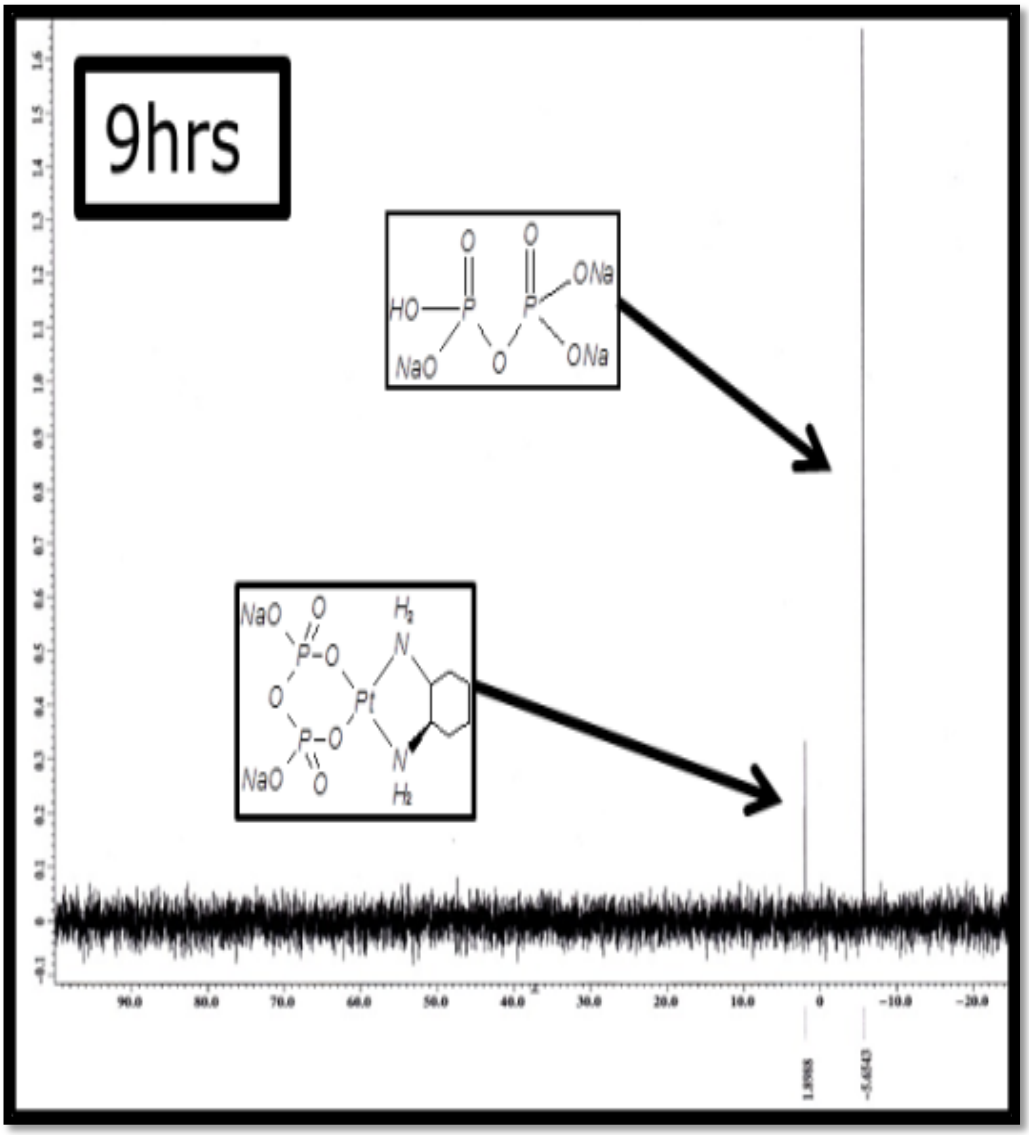
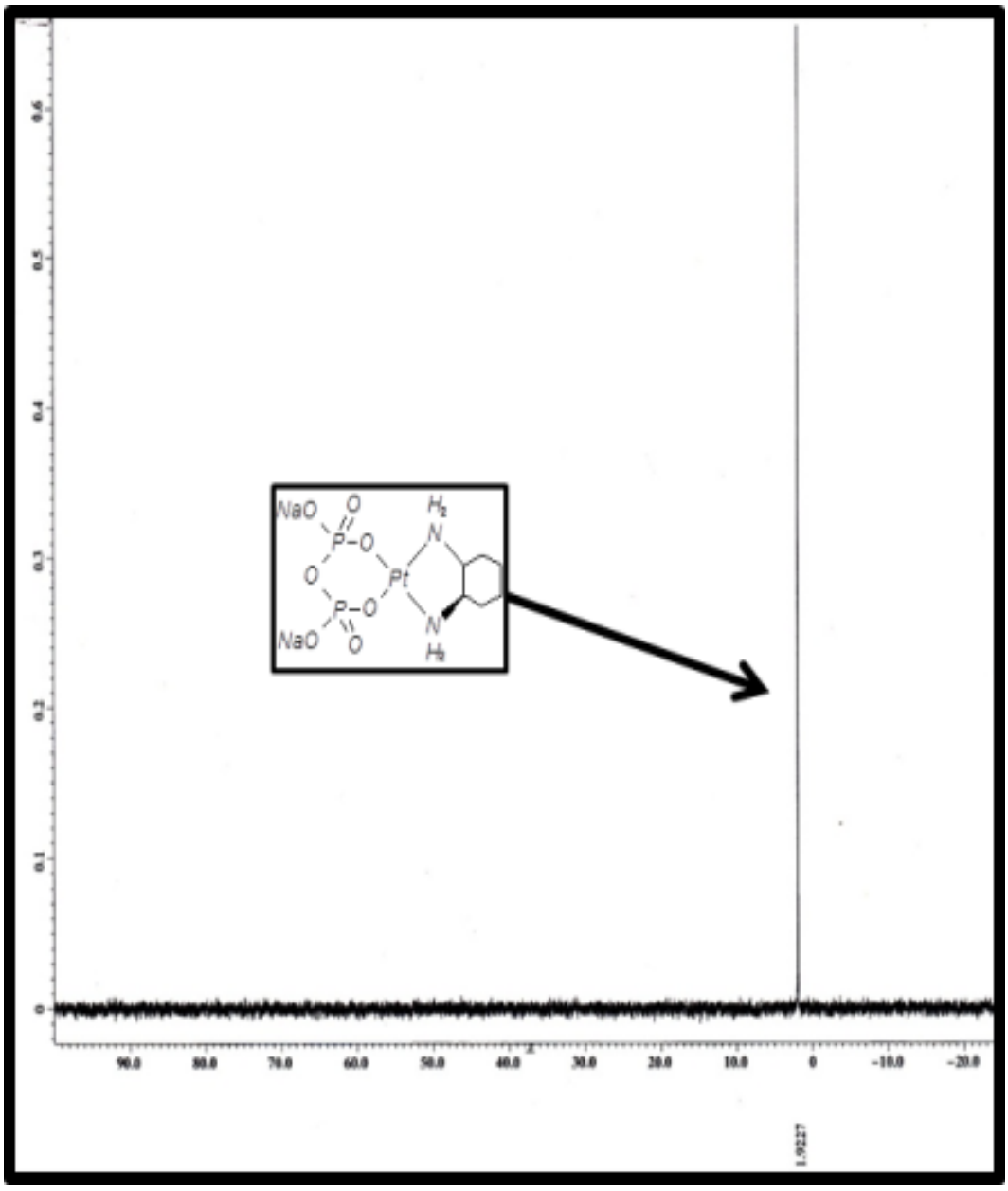

9hrs
HO
NaO
O
ONa
ONa
NaO
NaO
Pt
N
N
H2
H2
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0
-10.0
-20.0
1.9588
-5.6543

Figure 4:


NaO
O
P–O
O
Pt
P–O
NaO
O
H2
N
N
H2
0.6
0.5
0.4
0.3
0.2
0.1
0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0
-10.0
-20.0

As it is shown in Figure 5(a) high performance liquid chromatogram of the products recovered after the above first reaction cycle recorded immediately after dissolving the product (1 mg) in 600 μL of 25 mM sodium bicarbonate at pH 7.5. A 50-μL aliquot of the sample was injected for the separation. Each separation was repeated three times. The high performance liquid chromatography experiments were performed on a Waters HPLC system equipped with a dual gradient programmer and a photodiode array detector (Waters). Gradient separations were performed on a C18 column (Waters, XTerra R18, 4.6x150mm column, 5 micron) by using a mobile phase consisting of 10 mM ammonium acetate (pH 5.5) buffer (solvent A) and acetonitrile (solvent B). The gradient separation consists of linear increase of solvent B from 0% to 30% for the first 30 min followed by a steeper increase of 30 to 100% of B in the next 5 min. Finally, an additional 5 min of isocratic separation was set to 100% A. The flow rate was set at 0.5 ml/min throughout the gradient at room temperature. The respective peaks were collected at the beginning of each eluting peak to the end. Referring to Figure 5(a), the HPLC peaks at 3.04 and 4.2 min correspond to the deligated pyrophosphate ligand and the desired monomeric complex (phosphaplatin), respectively. We believe that the released ligand and the dimeric compound are formed due to dynamic behavior of the compound in acidic solution, which is absent at neutral pH where only the monomeric compound exists in solution(4).

The mass spectrometeric analysis of final product and collected HPLC fractions of the final product were performed on LCQ DECA-XP (Thermo-Finnigans) mass spectrometer by direct infusion using 500 μL syringe (2.30 mm diameter) at a 8μL/min flow rate (infused volume 32 μL). The mass spectrometer was set to positive ion

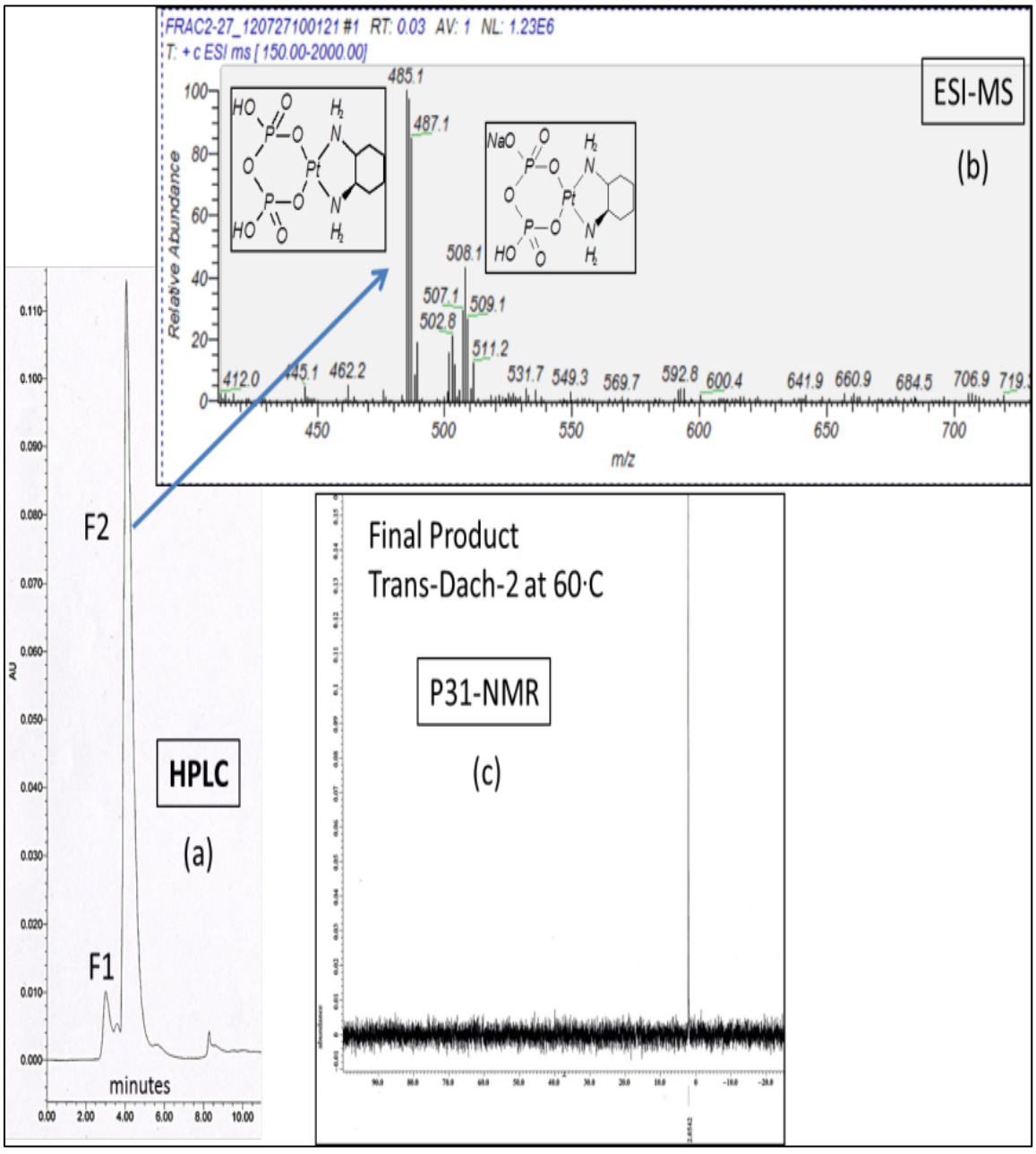
polarity (+MS), dry temperature of 350 °C, capillary voltage 30.22 V, sheath gas flow rate at 49.36 l/min, dry gas 5.00 l/min, and tube lens voltage 15.0 V. The mass to charge ratios was collected from 150 to 2000. The collected fractions were also identified via mass spectrometry. Referring to Figure 5(b), the peak at m/z 486.4 corresponds to the desired monomeric complex (phosphaplatin). The peak at m/z 508.1 corresponds to its sodium adduct (4).

NMR experiments were performed on a JEOL ECA-500 MHz instrument equipped auto-tune broadband n-15-P31 probe using the JEOL delta operation's software. Proton decoupled 31P resonances were recorded at 202 MHz and their chemical shifts are reported with respect to 85% phosphoric acid at 0.0 ppm. A pulse of 4.6-microsecond with a repetition time of 0.8 s was used to generate Fourier induction decay. Typically, 52 K data points were collected within 31.72 KHz frequency domain. A line-broadening factor of 1.0 Hz was introduced before Fourier Transformation. As shown in Figure 4(c), at pH 7.09, the P-31 NMR spectrum displayed a single peak at 2.02 ppm (trans-dach-2). The product exhibited a single P-31 NMR resonance at 2.02 ppm with two satellites due to coupling with Pt-195 nuclei that contain a nuclear spin of ½, as expected for a pure monomeric pyrophosphate-platinum(II) complex. Furthermore, when analyzed at pH 7.5, the P-31 NMR spectrum displayed a single peak at 1.92 ppm (RR-dach-2) (4).

The mother liquor left over from the isolation of the product was used for a second cycle of synthesis by replenishing 0.1 g pyrophosphate ligand along with the 0.1 g of the starting platinum complex. The exact same process explained above was followed to isolate the product. The yield of the product again was consistent with the first batch as

noted above. The HPLC chromatogram, mass spectra, and P-31 NMR of the product isolated in all of these experiments were the same.

FIGURE 5: Shows HPLC data for products recovered after performing a process according to an embodiment of the present invention.



3.2 *In vivo* and *in vitro* efficacies

In order to test the effectiveness of all the isomers of phosphaplatins *in vitro* against cisplatin-sensitive and resistant human ovarian cells, we performed a series of clonogenic assays. In those studies we have compared the survival rate of cells treated with phosphaplatins with those treated with cisplatin and carboplatin. Our results indicate that RRD2 complex shows superior IC_{50} value compared to two commercially available platinum compounds, cisplatin and carboplatin. The IC_{50} (μM) values calculated from the clonogenic assays in four different ovarian cell lines are listed in Table 1. These results help us to obtain the appropriate dose when treating the cancer cell lines.

These efficacy studies help us with *in vivo* experiments as well as *in vitro*. Based on the *in vitro* IC_{50} values and *in vivo* maximum dose tolerance, *in vivo* efficacies for the R,R-2 and R,R-4 were assessed against A2780 human ovarian cancers by implanting human xenograft in NIH mice .

Table 1

IC_{50} values of phosphaplatins, cisplatin (CP), and carboplatin (CR) in human ovarian cells determined by clonogenic assays by exposing platinum compounds for 24 h.

Cell line/ compounds	*R,R*-D2	*S,S*-D2	D-2	*R,R*-D4	*S,S*-D4	D-4	CP	CR
OVCAR10	0.42	6.9	4.6	10.2	–	14.4	4.1	26.7
OVCAR5	15.4	4.5	12.2	19.8	5.6	–	–	–
A2780	1.02	1.07	2.57	10.7	5.2	4	2	4.1
A2780/C30	6.9	-		16.5	–			

3.3 Chip microarray

To further study the mechanism of action of phosphaplatins, we had to figure out what pathways were affected during the treatment with phosphaplatins, and which genes were involved in those pathways.

Cells were treated with different concentrations of RRD2 and RRD4 for 12 and 24 hours. The total RNA from the treated human cells was extracted and the concentration and integrity of all RNA samples were assessed. 1ug of total RNA from each sample was subjected to whole-genome gene expression analysis at the Microarray Core Facility of University of Texas Southwestern Medical Center (https://microarray.swmed.edu) using the HumanHT-12 v4.0 BeadChip (Illumina, San Diego, CA) as explained in the Methods section in chapter 2 and the results were interpreted using SAbiosciences to find the relation ship between these genes.

In our analysis of chip microarray results, we have focused mainly on the genes that were significantly up regulated or down regulated with phosphaplatin treatments. As we have mentioned previously, phosphaplatins do not bind to DNA, they only interact with proteins. Therefore, our results should be confirmed with a series of western blots by examining these protein expressions. So, we have mapped one of the pathways for a group of these genes, which were significantly affected by phosphaplatin treatments.

The expressed genes in 24hr treatment show a close relationship of TP53 signaling pathway with neighboring genes to be one of the major mechanistic pathway of RRD2 and RRD4 in the treatment of A2780. We have also provided a list of these genes and their fold change with the treatment of both RRD2 and RRD4.

Figure 6: gene relationship for the treatment of A2780 with RRD2 and RRD4.

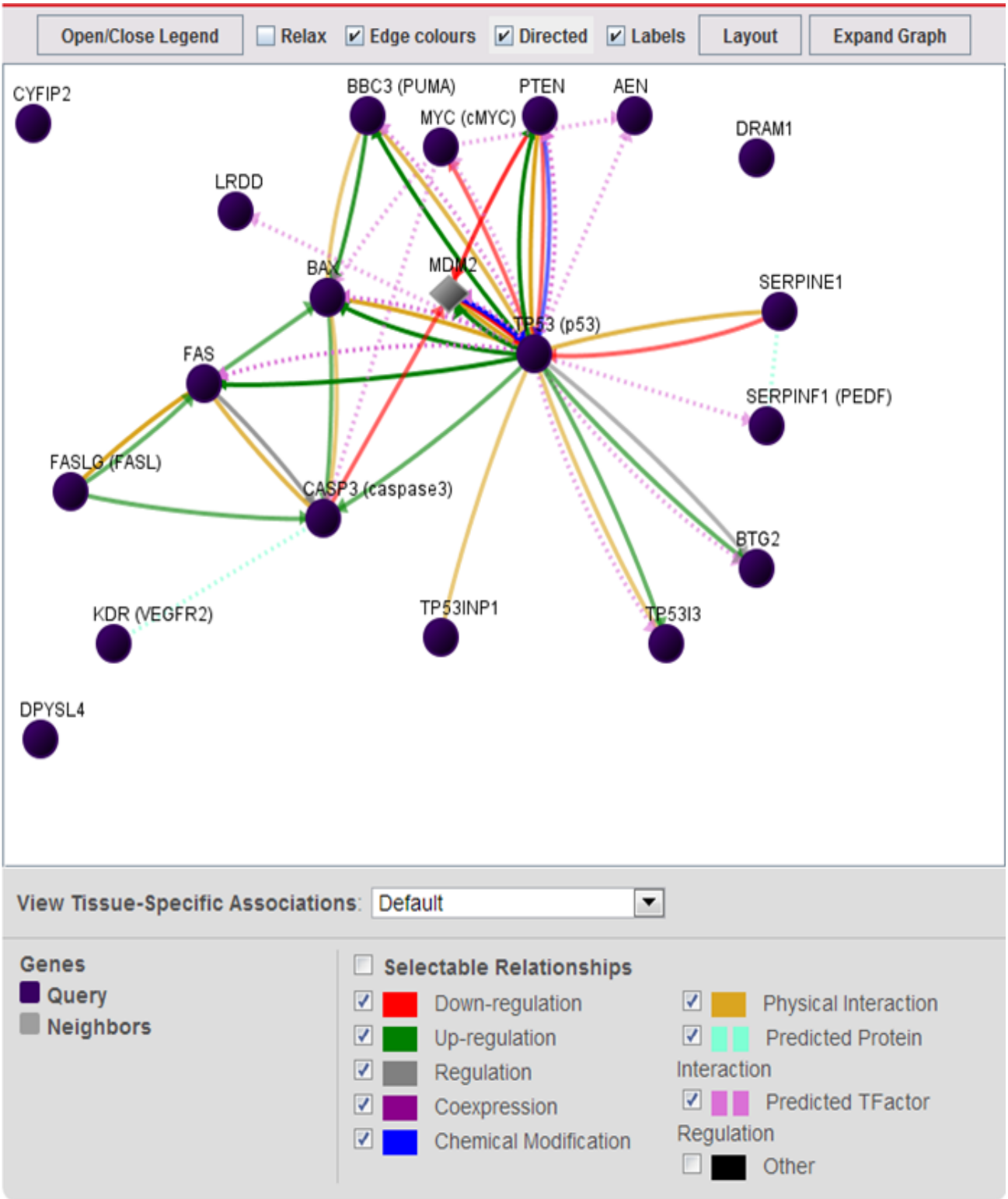


Table 2: Fold change in genes due to treatment with 10 μM RRD2 and 25 μM RRD4 for 24 hour.

SYMBOL	**10RRD2-24hr Fold Change**	**SYMBOL**	**25RRD4-24hr Fold change**
GADD45A	**2.23**	GADD45A	**1.54**
BAX	**2.60**	BAX	**2.59**
BTG2	**4.37**	BTG2	**2.60**
CYFIP2	**3.29**	CYFIP2	**2.40**
DPYSL4	**3.03**	DPYSL4	**1.96**
DRAM1	**2.16**	DRAM1	**1.81**
LRDD	**1.63**	LRDD	**1.53**
FBXO22	**1.85**	FBXO22	**1.48**
TP53I3	**5.66**	TP53I3	**3.62**
TP53INP1	**2.62**	TP53INP1	**2.08**
SPATA18	**2.13**	SPATA18	**1.84**
SERPINE1	**1.53**	SERPINE1	**1.27**

3.4 Apoptotic Properties of Platinum Antitumor Agents Phosphaplatins

To further characteize RRD2 and RRD4 compounds and to determine their mechanism of action, the examination of protein expression became important after gene expression analysis. As discussed in the chip microarray data section of this chapter, RRD2 and RRD4 anticancer agents were able to regulate and activate apoptotic and pro apoptotic genes such as BAX, PUMA, FAS, P53, DR3, DR5, BAD, CASPASE3, CASPASE8, Bcl2, FADD at the transcriptional level. The regulation pattern of these genes indicates that their protein expressions must play an active role in inducing the pro-apoptotic signals leading to cell death.

To examine the protein expression level of these genes, we have treated A2780 cell lines with RRD2 and RRD4 at 1 and 12 hr and measured the expression of these genes.

Figure 7: 12 HR treatment with 10RRD2 and 25 RRD4

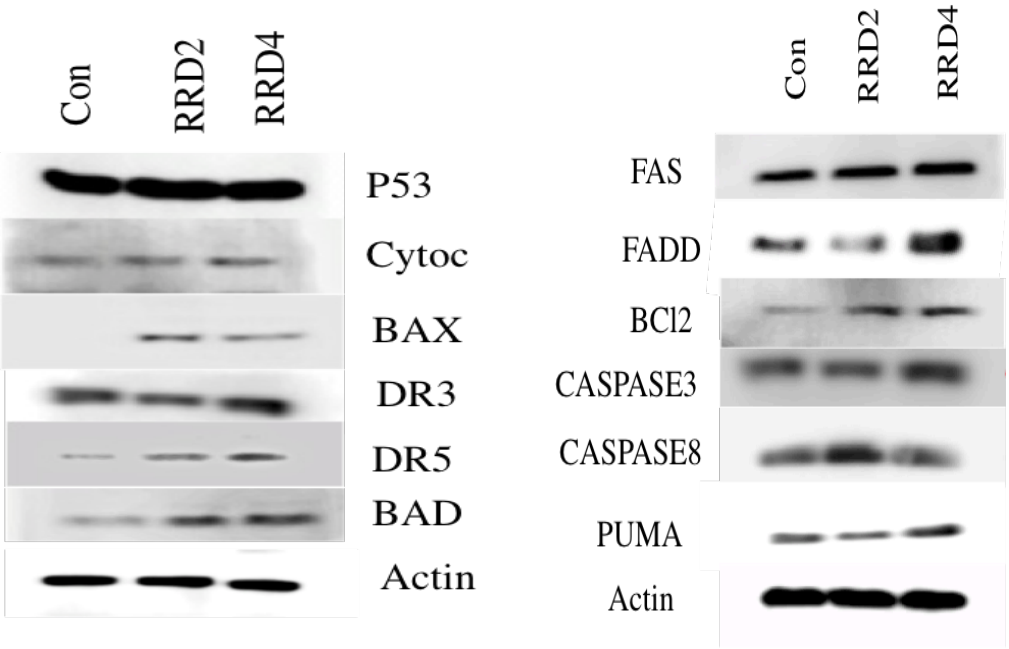


Figure 8: 1 HR treatment with 10RRD2 and 25 RRD4

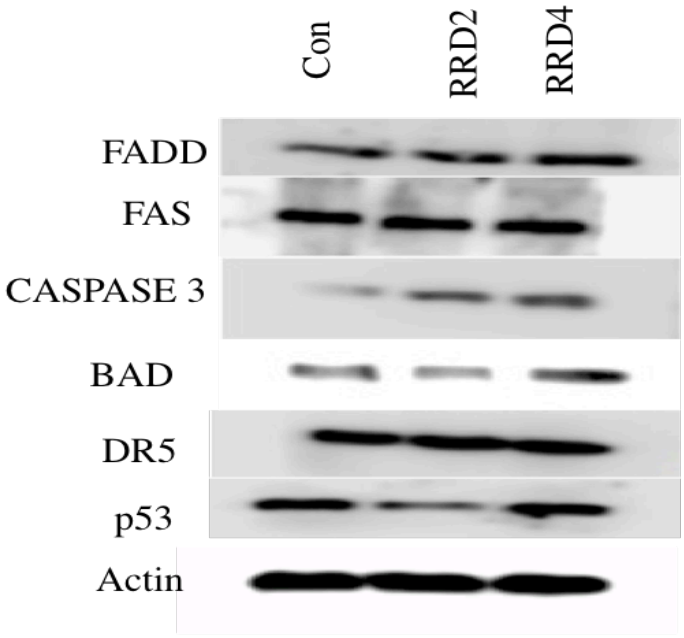
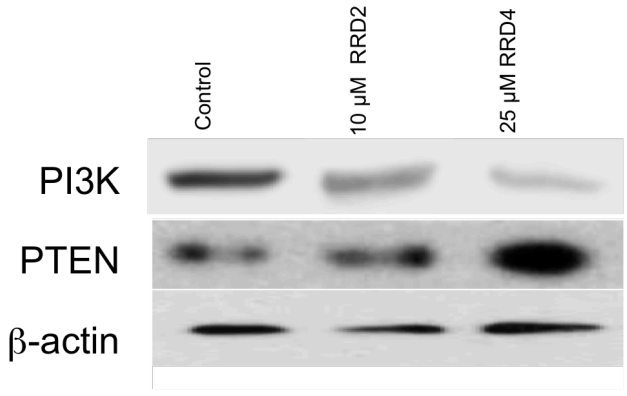
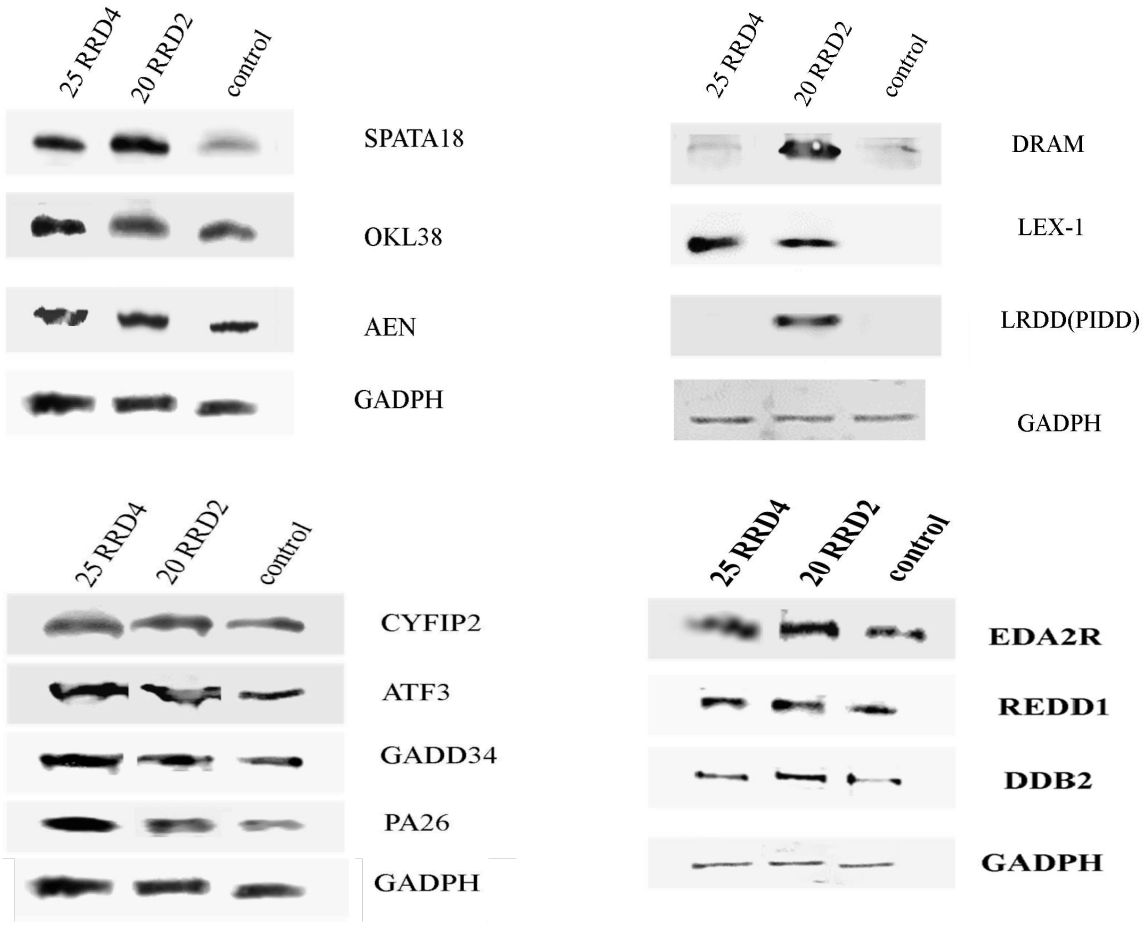


Figure 9: Representative Western blots showing phosphaplatin effects on proteins isolated from A2780 versus untreated controls. Specifically we show representative immunoblots of PI3K protein expression at 3 hrs and PTEN protein expression at 6 hrs, confirming PTEN-PI3K pathway involvement.



As it was shown in the chip microarray data, a group of P53 related proteins were also overexpressed. In order to confirm our results, we have performed western blot for 12 hrs.

Figure 10: A2780 cell lines were treated with 20 μM RRD2 and 25 μM RRD4 for 12 hours. All of these proteins are overexpressed in response to treatment with phosphaplatins.



3.5 Phosphaplatins as neuroprotective agents

It is known that Pigment Epithelial Derived Factor (PEDF) is a potent anti-neovascular or anti-angiogenic agent as well as a neurotrophic factor. It has also been shown that the expression of PEDF is associated with neuronal differentiation and survival factor for cells derived from the central nervous system (CNS) and retina. PEDF has neurotrophic effect on neurons from areas including the spinal cord, hippocampus and cerebellum. Neurotoxicity due to cancer treatment is often associated with administration of chemotherapeutic drugs.

We found that PEDF gene is over expressed in human cancer cell lines that are treated at different time courses using phosphaplatins antitumor agents. Individual gene expression for PEDF was assessed by two-step qRT-PCR assay done by Dr. Shadi Moghaddas.

Table 3: Fold change expression of PEDF in A2780 after treatment with RRD2 and RRD4 , calculated by ΔΔCT method vs. the endogenous control β-actin and/or GADPH.

Compound (treatement)	Fold Change (±SD)	Method of Analysis
5uM RRD-2 (24 hr)	2.64	Angiogenesis Array
13uM RRD-4 (24 hr)	2.05	Angiogenesis Array
20uM RRD-2 (24 hr)	5.79	Angiogenesis Array
50uM RRD-4 (24 hr)*	4.1	Single gene expression
25uM RRD-4 (3hr)*	2.1	Single gene expression

* endogenous control GADPH was used instead of β-actin

According to Table 3 PEDF has been significantly overexpressed when treated with 20 μM RRD2 and 50 μM RRD4 for 24 hours. In order to examine the expression of PEDF at different time points, we have treated the ovarian cancer cell line A2780 for 3, 6 and 12 hours as well and assessed PEDF overexpression by IF and western blot experiments.

In figure 11, we can see that PEDF is significantly overexpressed when treated with 25 RRD4 at 6 and 12 hr. Our IF experiments have also confirmed our western blot results.

Figure11: PEDF has shown to be upregulated in response to treatment with phosphaplatins at 6 and 12 hr.

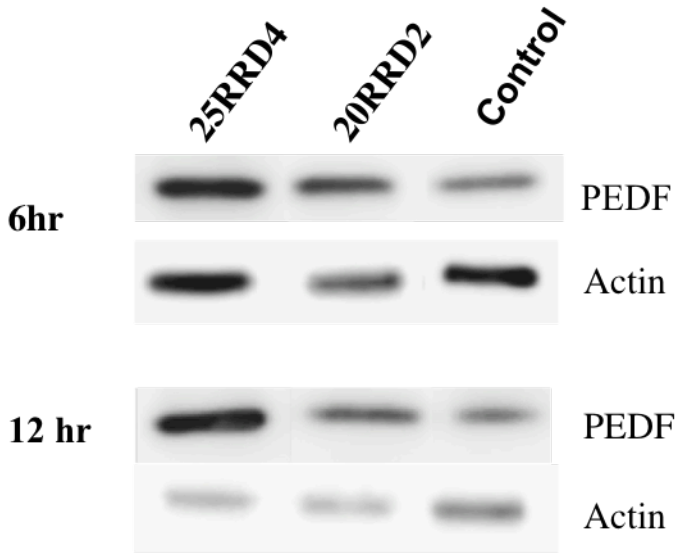


Figure 12: Overexpression of PEDF demonstrated by IF from 12 hr phosphaplatin treatments.

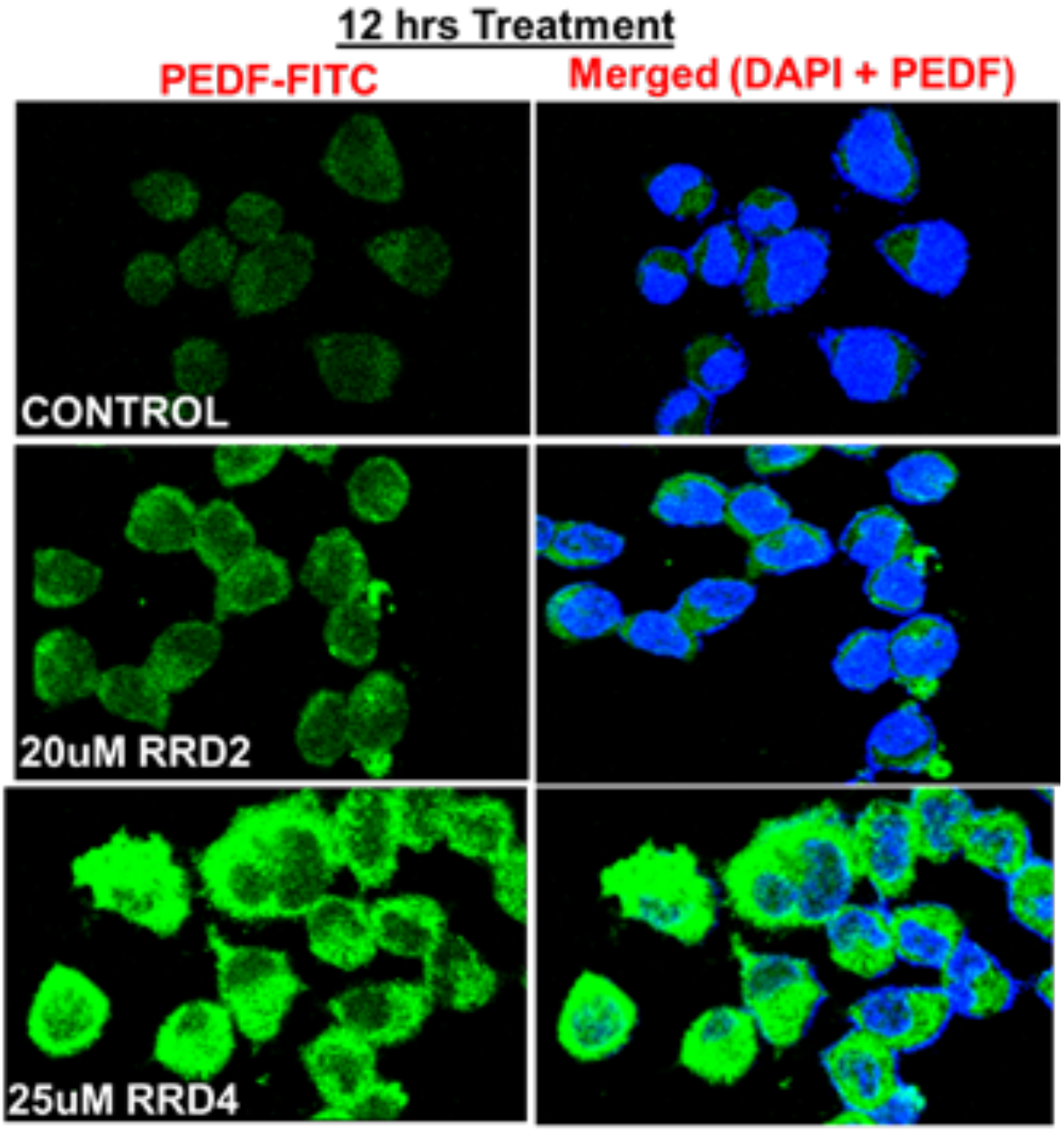


Figure 13: Over expression of PEDF demonstrated by IF from 6 hr phosphaplatin treatments.

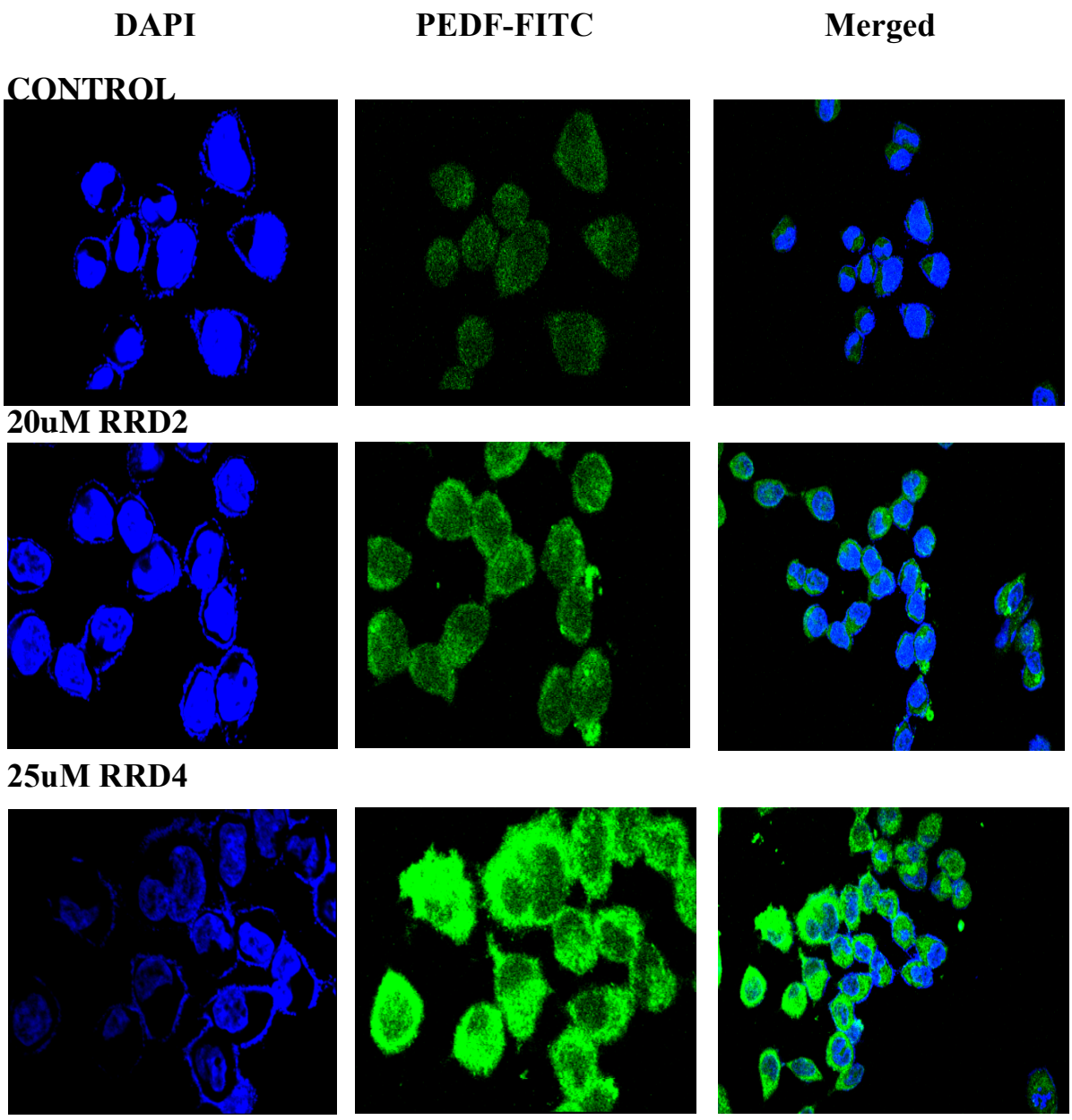
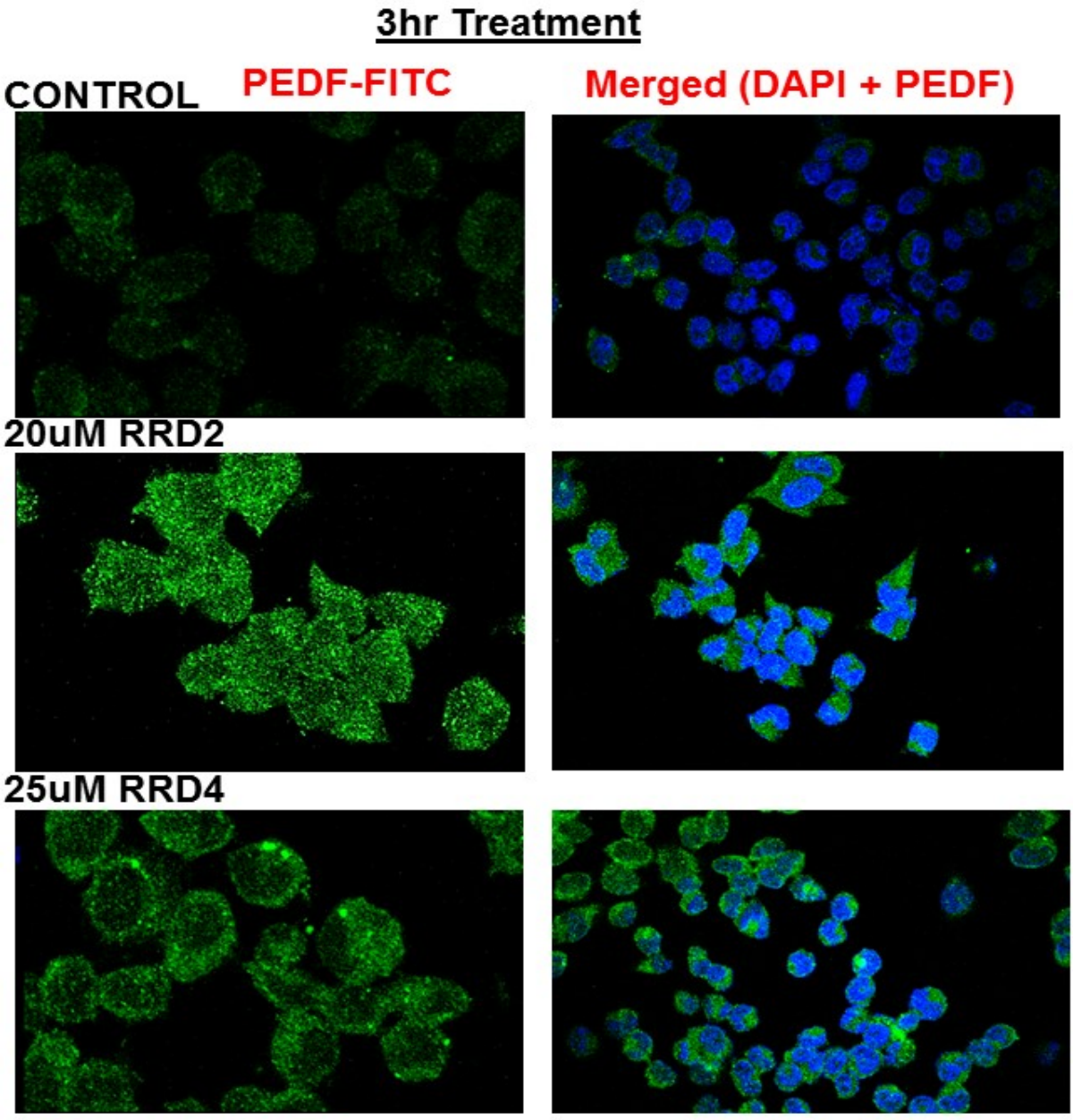


Figure 14: Over expression of PEDF demonstrated by IF from 3 hr phosphaplatin treatments.



3.6 Preclinical animal studies

To investigate and identify the potential drug treatment options for cancer therapy, we have done a series of preclinical animal studies. In the early stages of drug development, we have conducted toxicology studies using rodents to safely predict and establish a range of toxicities and to determine the maximum tolerated dose (MTD) for further characterizing the phosphaplatin compounds.

In order to assess the toxicity and maximum tolerated doses of phosphaplatins a series of experiments were performed. One of these experiments was a thirty-day dose tolerance study with a three-single dose regimen administered on days 1, 3, and 5 with doses ranging from 10 mg/kg to 100 mg/kg. These doses were chosen based on the results from the IC50 values.

In this study we have compared different dosages of RRD4 to Cisplatin and PBS control group. As can be seen from the data in Figure 13, RRD4 was well tolerated at doses up to 60 mg/kg while 80% of cisplatin treated mice died at a dose of 10 mg/kg.

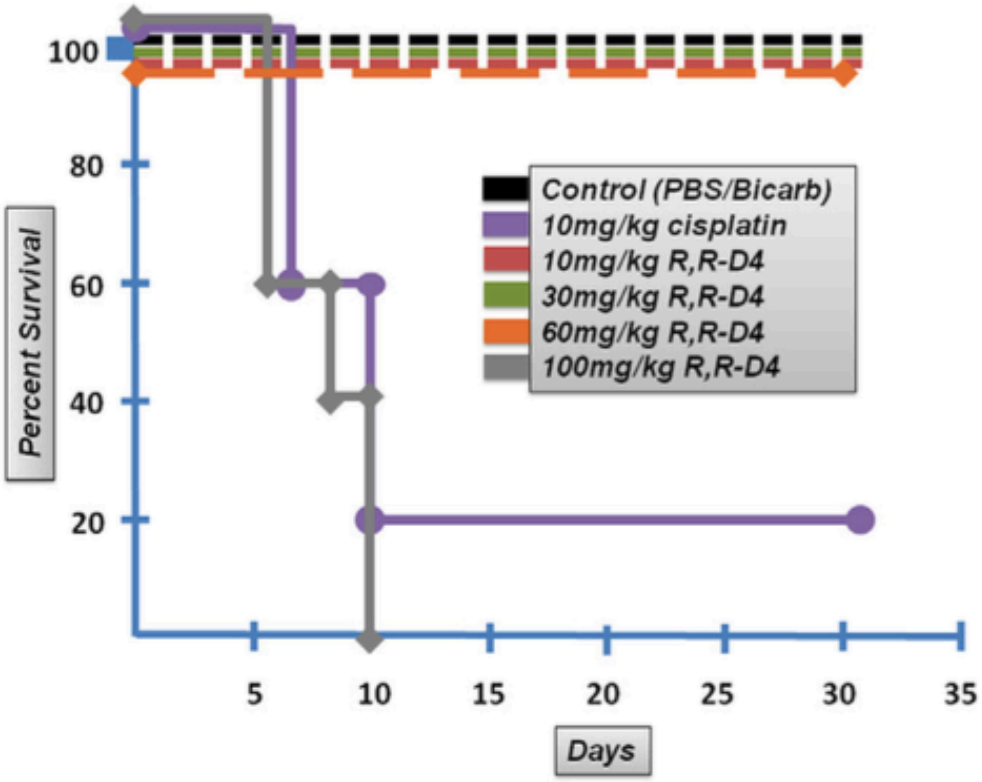


Figure 15: Mice survival curves monitored for 30-days after administering cisplatin (10 mg/kg), and R,R-D4 in various doses ranging from 10 to 100 mg/kg.

Next we have conducted a seven-day toxicity study where blood chemistry was monitored at day 1, 3, and 7 after administering a single dose (30 mg/kg) of R,R-D2 and R,R-D4. The white blood cells, neutrophils, and platelet counts were found to be comparable to the control group up to 30 mg/kg doses. A small decrease in platelet count was observed initially and then a quick recovery was observed within a few days. Table 4 shows the counts of various markers.

Components	Control	*R,R*-D4	*R,R*-D2
WBC (K/μL)	3.5(0.6)	5.4*(0.7)	4.4(0.7)
NE (K/μL)	1.1(0.2)	1.7(0.3)	1.1*(0.3)
PLT (K/μL)	821(62)	669*(31)	609*(126)

* Represents *p* values <0.05.

Table 4: Blood analysis from peripheral blood collected from terminal cardiac puncture after 24 h post-treatment to CD1 Mice (N = 5 each group) with R,R-D2 (30 mg/kg) and R,R- D4 (30 mg/kg).

Lastly, we have performed a single dose experiment to determine platinum content in plasma, liver, kidney, and urine within the first 24 h after administering the anticancer agents. Figure 16 shows the platinum content in the kidney tissue at a dose of 10 mg/kg. Note that the platinum content decreased from 4 ug/g to below 1 ug/kg within 24 hrs.

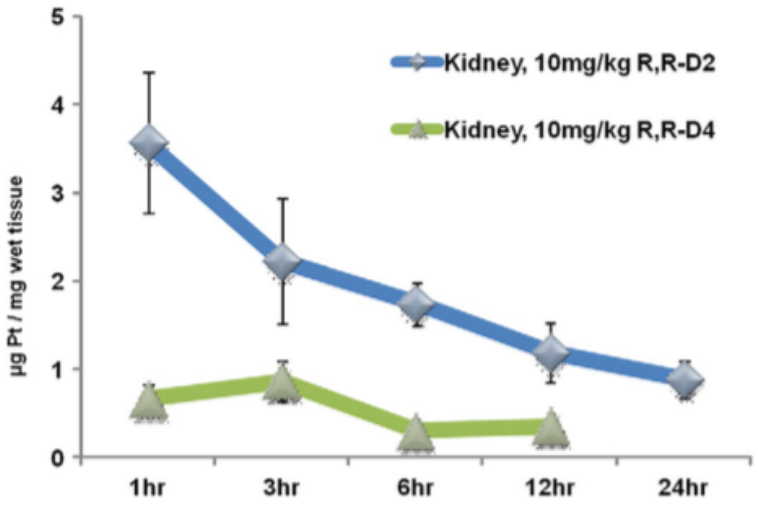


Figure 16: Accumulation of platinum in kidney of CD1 mice after injecting R,R-D2 (10 mg/kg). Each time point is an average of five animals.

As shown in Figure 17, the tumor growth curves for the phosphaplatin complexes and cisplatin in A2780 xenograft. Both of the phosphaplatin compounds R,R-2 and R,R-D4 show superior efficacies compared to cisplatin. The ILS values for phosphaplatins were between 130% and 150% while cisplatin merely exhibited 40% ILS. Likewise, phosphaplatins either show stasis or regression while cisplatin barely shows inhibition.

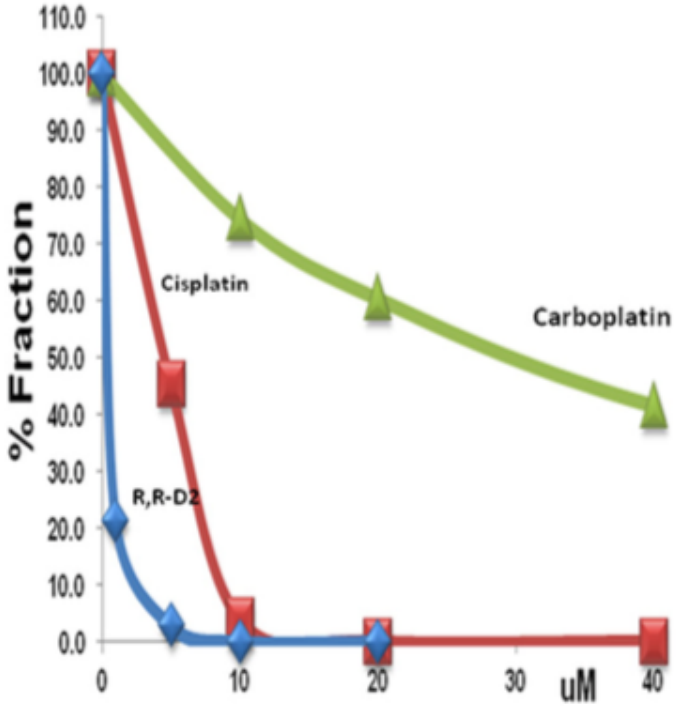
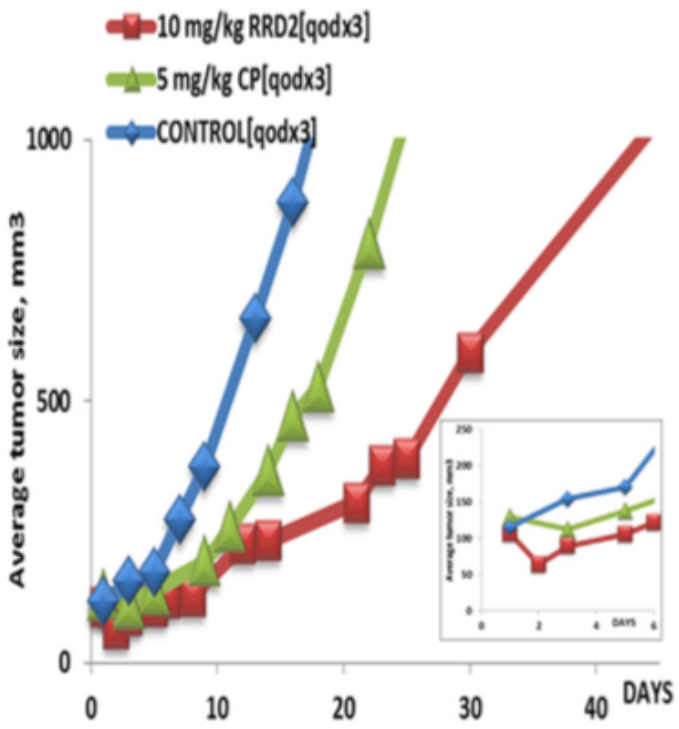


Figure 17: Top panel: Cell survival curve for cisplatin-resistant human ovarian cancer cells (Ovcar-10) after treating with various concentrations (uM) of cisplatin, carbopaltin, and R,R- D2. Bottom panel: *In vivo* efficacy of cisplatin (7 mg/kg) and R,R-D2 (10 mg/kg dose) in human xenograft model (Ovcar-10) determined by implanting Ovcar-10 in NIH SCID mice and treating three doses of the platinum agents. The inset in the bottom panel indicates tumor regression right after treatment for R,R-D2 while inhibition for cisplatin.

Fig 17:


10 mg/kg RRD2[qodx3]
5 mg/kg CP[qodx3]
CONTROL[qodx3]
Average tumor size, mm3
1000
500
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4 DISCUSSION

Over the past two decades, researchers have been trying to optimize and improve the quality of cancer treatments. The standard treatment of ovarian cancer is a combinatorial treatment, which consists of cisplatin or carboplatin and taxol.
Unfortunately, due to acquired or inherent resistance towards these platinum drugs a significant number of patients develop resistance or does not respond to the chemotherapy. We have designed a new non- DNA binding platinum compound that has less toxicity and can overcome both acquired and inherent resistance to treatment.

Based on the results that we have presented in previous sections, we can conclude that phosphaplatins show remarkable efficacy and reduced toxicity compared to other platinum compounds. This superior efficacy and reduced toxicity could be due to the different cellular and molecular antitumor mechanism of pyrophosphate complexes. One of the major differences between phosphaplatins and other commercially available compounds is the significantly lower IC50 values. According to our results RRD2 has a substantially lower IC_{50} value compared to cisplatin. However, all of our compounds have much lower IC50 values compare to carboplatin and cisplatin, which is , at least ten-times lower when assessed in the 24 hr treatment studies. Our results conclude that phosphaplatins can treat resistant ovarian cancers significantly better than other antitumor agents.

Next, we also conducted a series of *in vivo* animal studies by directly comparing phosphaplatins with cisplatin. Our data suggested that the animal group that was treated with the phosphaplatin compounds have an increased life span and reduced tumor size.

The maximum tolerated doses for phosphaplatin compounds is much higher than cisplatin but comparable to carboplatin. On the other hand, the therapeutic window for phosphaplatins is much larger than carboplatin, and they are more efficacious than carboplatin. The efficacy data that we obtained from carboplatin with 60 mg/kg dose is inferior to 10 mg/kg dose of R,R-D2.

One of the most important issues with chemotherapy is its toxicity. We have measured the accumulations of platinum in kidney and other organs. Based on our results we can conclude that phosphaplatins are significantly less toxic to the kidney and other key organs, partly becausethey accumulate less than cisplatin in these organ tissues. The amount of cisplatin accumulated in the kidney tissue for one hour was as high as 10 ug/g and after 24 hr over 4 ug/kg retained in the kidney. Whereas, after 24 h, R,R- D2 and R,R-D4 showed below 1 ug/kg retention of platinum in the organ. This reduced toxicity and low dose of phosphaplatins can make the treatment more tolerable for patients who cannot tolerate high doses of platinum therapeutics.

Studies have indicated that protein binding to platinum anticancer drugs can inactivate their antitumor activity. Only 1–2% of cisplatin binds to nuclear DNA and more than 75% of administered platinum compounds are usually bound to protein and small molecules. [25,26].

In conclusion, *in vitro* and *in vivo* data indicate that phosphaplatins exhibit superior efficacy and reduced toxicity compared to several other commonly used platinum-based compounds that are currently in use to treat ovarian cancers.

The proposed antitumor mechanisms of RRD2 and RRD4 are illustrated in Figure 18. The key points are:

1. *In vitro* and *in vivo*, superior efficacy of RRD2 and RRD4 for the treatment of human ovarian cancer cell line, A2780 compared to commercially available Pt compounds on the market (DNA-binding agents : cisplatin, actinomycin D, doxorubicin, and bleomycin) with no toxicity and adverse side effects.

2. Phosphaplatins do not form covalent linkage with DNA and the administered compound is largely distributed in the extracellular matrix and cytoplasm and not the nucleus (data not shown), the mode of action of this class of compound must be different from conventional platinum therapeutics.

3. Our data showed that phosphaplatins triggered up regulation of PUMA, P53, BAX, Fas, FasL and PTEN in human ovarian cancer cells, A2780. A number of apoptotic and tumor suppressions proteins are involved in participation through extrinsic and intrinsic pathways via FAS.

4. FAS activation leads to the engagement of DISC formation through recruitment of FADD and procaspase-8 leading to the activation of Caspase 3 and followed by the apoptosis of the cells.

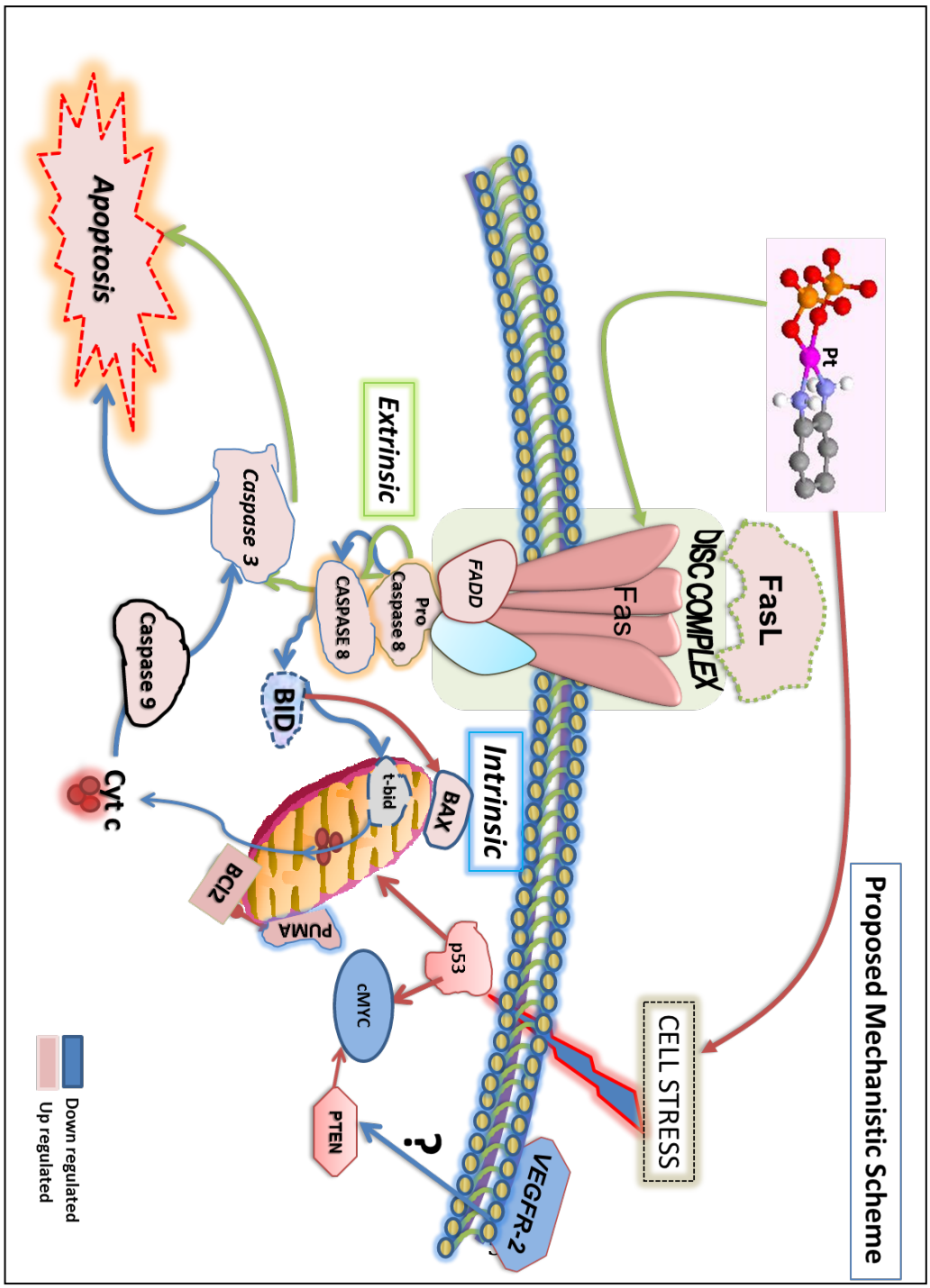
5. Phosphaplatins have been shown to involve the mitochondrial apoptosis pathway that is triggered by the overexpression of BAX and Bid and the downregulation of

the proapoptotic BCL2,hence, leading to the release of cytochrome c into the cytoplasm by the binding of localized PUMA in the mitochondria to BCL2 and exerting its apoptosis effect.

6. Even though PUMA is a P53 dependent transcription factor, based on recent studies it is capable of mediating apoptosis through a P53 independent pathway. Phosphaplatins have triggered up-regulation of Fas, FasL, Bax, p53, PUMA, and PTEN in A2780 cells.. In addition over expression of P53 to regulates the expression of PTEN at the transcriptional level and or possible post-transcriptional levels. However, it is to be elucidated that the cross talk between P53 and PTEN even though there is no significant enhancement of cell death observed in the presence of the PI3K inhibitor and in the presence of α-pfifthrin, a p53 inhibitor (data not shown).

7. PTEN expression was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency and by inhibiting PI3K and Akt. Uncontrolled growth of malignant cells triggers angiogenesis, while PTEN has been shown to possess the antiangiogenic property. We have shown that phosphaplatins can upregulate PTEN and PDEF to exert its anti-angiogenetic acitivity. Furthermore, phosphaplatins have shown to lead to the over activity of p53 over-expression that has been followed by the regression and loss of c-Myc regulation in tumor suppression.

Figure 18: Proposed mechanism of action for RRD2 and RRD4



In conclusion, phosphaplatins exhibit a paradigm shift in signaling mechanisms when compared to conventional platinum cancer chemotherapy. To the best of our knowledge, phosphaplatins are truly unique as no small molecules reported to date activate a combination of apoptotic and tumor suppression genes observed here through parallel signaling as a mono-therapeutic agent.

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