Copper Tolfenamic Acid as a Novel Survivin Inhibitor

for Suppressing Pancreatic Cancer Cell Growth via Downregulating

Sp1 and Sp3 Transcription Factors

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSPHY

By

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June 4th, 2018

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ACKNOWLEDGEMENTS

First, I would like to express my immense gratitude to my mentor, Dr. Riyaz Basha. His guidance, patience, and vast knowledge helped me develop the skills required to complete my PhD. I appreciate all of your advice, time and support during my graduate school experience. It has been a privilege working in your laboratory.

For all of their suggestions and direction throughout my project, I would also like to thank my committee members Dr. Andras Lacko, Dr. Yi Qiang (Eric) Cheng, Dr. Porunelloor Mathew, and my university member Dr. Rong Ma. I am also extremely appreciative of Dr. Umesh Sankpal for all of his help and patience during my time in the lab. A special thanks to Dr. Alvin Holder for his collaboration and providing the investigational drug of my project. I am also grateful for Dr. Laszlo Prokai for his contribution and guidance with one of my project's aims. I extend my thanks to my graduate advisor, Dr. Alakananda Basu, and department chair, Dr. Paul Bowman, for their guidance and advice throughout my PhD. For all of their time and help with flow cytometry, I also give thanks to Dr. Xiangle Sun.

I would also like to express my appreciation to all of my lab members, both past and present, for providing a stimulating environment and working together to learn the skills needed in the research field of science. I am also grateful to our interim executive director of student services, Carla Johnson, for her constant assistance and support. Additionally, I would like to acknowledge and thank our administrative staff for all their help and assistance, including Jacklyn Crisp, Lenore Price, Deanna Ranker, and Jessica Medina.

Additionally, I am thankful for the financial assistance supported by a grant from the Shirley E. Noland Foundation.

Finally, I wish to thank my friends and family for their encouragement. Especially my fiancé, Sunil, for his love and support. They all provided me with the strength to successfully complete my PhD.

Sincerely,

Myrna Hurtado

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER – I

BACKGROUND LITERATURE

INTRODUCTION: CANCER

Cancer is a disease that has been afflicting people since humanity began. There have been several documented cases describing tumors that date back to thousands of years ago with the earliest documentation being around 3000 BC [1]. Unlike most who credited these masses of tissue to be a result of supernatural forces, the Greek physician Hippocrates believed it was due to natural causes [2]. Hippocrates coined the term carcinoma because the way malignant tumors spread reminded him of a crab [3]. Since little was known about cancer, if the disease was too advanced then it would typically be deemed untreatable. Other remedies included herbs, teas, surgery or cauterization [4]. Since then, there have been major medical advancements to significantly improve and extend our lifespan. The discovery of carcinogens, cancer viruses, emergence of early detection methods, classification of tumors, and chemotherapy and radiation as effective therapies drastically changed our ability to diagnose and treat patients [5-9]. Although cancer research has made tremendous progress in the past 50 years, cancer is still considered to be the plague of the 21st century. It is estimated that about 1.7 million people were diagnosed with cancer last year in the United States alone. 600,000 of which are predicted to have died from cancer related disease [10].

Cancer arises due to an accumulation of mutations that lead to the cell's continuous proliferation. However, it has been widely accepted that cancer is not merely uncontrollable proliferation. It is a multifactorial disease with a myriad of aspects that all contribute to its development and advancement. There are many defining characteristics to cancer including

continuous proliferation by sustained self-sufficient secretion of growth signals, evasion of apoptosis, unlimited replicative potential, altered metabolism, angiogenesis, tissue invasion and metastasis, and escaping the immune system [11-14]. Cancer cells have been discovered to be self-sufficient in sustaining their own continuous proliferation. They secrete growth factors and by autocrine and paracrine signaling, are able to support their own and neighboring cells' growth [15]. Cancer cells are also able to evade apoptosis, thus ignoring the cell's genomic instability and other signals that would normally alert the cell to undergo self-programmed cell death [16-18]. Avoiding apoptosis allows more mutations to arise, which is needed when selective pressures such as immune detection become an issue for cancer cell survival. Immune cells such as neutrophils and regulatory T cells are responsible for detecting and removing cancer cells when they appear in the body [19]. Cancer immunoediting is another defining characteristic. The cells that are able to escape recognition (perhaps by lacking tumor-antigens or by directly suppressing immune function by secreting certain cytokines) are selected for because they are able to continue replicating [20-22]. Of course, there are many more aspects involved with cancer beyond what was mentioned, but even so it is apparent that this is an incredibly complex disease. This complexity is what makes finding a cure so difficult.

Pancreatic Cancer

Pancreatic cancer (PaCa) arises when there is uncontrollable cell proliferation that originates in the pancreas and forms a tumor. This tumor disrupts the normal pancreatic cell function and more often than not, metastasizes to nearby organs such as the gallbladder, liver, and stomach [23]. The pancreas is a small fish-shaped organ about six inches long located behind the lower part of the stomach. The pancreas is mainly comprised of two cell types: exocrine and endocrine cells. The exocrine cells are responsible for synthesizing and secreting enzymes into the small intestine to aid digestion [24]. The endocrine cells release insulin and glucagon into

the bloodstream to regulate blood glucose levels in the body [25]. More than 95% of PaCa cases are determined to be exocrine tumors [26]. The exact cause for a person to develop PaCa is difficult to determine. However, there have been several risk factors identified to contribute to development. Cigarette smoking is the top preventable risk factor for PaCa, which more than doubles a person's probability for development [27]. Other preventable influences include alcoholism, obesity, and unhealthy diets [28]. As with any cancer, age is considered to be a factor as well, due to the accumulation of mutations over a person's lifetime. Genetics are also deemed to be a risk factor [29, 30]. There are several cancer syndromes for PaCa that can be genetically inherited and thus increase a person's likelihood of development.

While many types of cancer have made considerable improvements in their overall survival rate and advancements in treatment, PaCa is one of the few that continues to be a fatal malignancy [31, 32]. Currently, the overall five-year survival rate is a dismal 8% [10]. PaCa is considered to be a lethal malignancy due to a myriad of reasons. The average age a person gets diagnosed with this disease is 65 years old. Due to their age, they usually have co-morbidities and are generally in poor health overall [33, 34]. There are also no early detection methods to date. There have not been any established biomarkers in the blood or bodily fluids. Unlike prostate, breast, or skin cancer, there aren't any physical examinations that can be performed due to the pancreas' location in the abdomen [35]. There have been some pancreatic antigens proposed to be used a as a potential screening, unfortunately there haven't been any discovered to be specific to only PaCa [36, 37]. Consequently, a diagnosis is often made in the later stages of this cancer at which point the disease is advanced. Additionally, this disease is normally asymptomatic during the early stages. In the late stages, there may be symptoms present, however they are generally non-specific (nausea, abdominal pain) [38]. Thus, this also contributes to its late diagnosis.

Approved treatment options for PaCa are currently limited in terms of selection and effectiveness. Standard treatment is typically chemotherapy (usually a combination of chemo agents) and radiation. Chemotherapy drugs for PaCa include gemcitabine, 5-fluoruracil, cisplatin, paclitaxel, and erlotinib [39, 40]. These cytotoxic therapies have a number of negative side effects such as vomiting, hair loss, low blood count and peripheral neuropathy [41]. Surgery is another treatment option; however, this approach is not feasible to more than 80% of patients [42-44]. This is again attributed to the fact that when patients begin treatment, the cancer is often at an advanced stage and has spread to other local organ sites. Another aspect of PaCa that adds to its fatality is the cancer cells' inherently or acquired resistance to treatment on both a physiological and molecular level. On a physiological level, creation of the tumor microenvironment adds to treatment resistance. A prominent characteristic of PaCa is the desmoplastic reaction. Desmoplasia is due to in an increased production of extracellular matrix components and activation of pancreatic stellate cells, both of which leads to elevated interstitial fluid pressure [45-47]. This increased pressure effects the perfusion rate of the chemotherapeutic agents, ultimately resulting in decreased molecular delivery and effectiveness. The genetic alterations in the PaCa cells also contribute to its resistance. While not all mutations that occur are understood how they result in chemo resistance, some mutations cause deficiencies in transporters required for drug uptake, thus impairing delivery [28, 48]. The resistance to chemotherapy and radiation poses an issue for patients since neither dosage can be increased due to the high level of toxicities [49, 50]. This provides an urgent need for improved alternative treatment options for PaCa that are more sensitizing, effective and less toxic.

Alternative treatment options for PaCa have been gaining popularity as it has become apparent that having a cytotoxic effect on the patient's cancer cells is just as important as not affecting the healthy cells. Immunotherapy is a prime example of this. Immunotherapy is harvesting the body's immune system to either boost the body's response or to help it specifically target cancer cells [101]. The benefits to immunotherapy are its low toxicity, being personalized for each patient, and an ability to have life-long effects on the immune system [102]. Having a treatment that is tailored to the patient is crucial for having the most effective therapy. The fact that immunotherapy can have long lasting effects on the immune system is also an important aspect since PaCa has over a 70% reoccurrence rate [103]. This form of treatment has been successfully used in other types of cancer cases and spiked interest in using it for pancreatic cancer. In 2004, a pancreatic cancer vaccine, GVAX, was created [104]. This vaccine was created by taking tumor cells expressing the protein granulocyte–macrophage colony-stimulating factor (GM-CSF) and irradiating them. Since GM-CSF is able to stimulate an immune response, these cells had been modified to express proteins found on pancreatic cancer cells to help the immune system specifically target the cancer cells. The concept of immunotherapy for PaCa was brought up and currently more promising and effective vaccines are being created and tested to better treatment [105].

POTENTIAL THERAPEUTIC TARGETS

Targeting aberrantly regulated transcription factors has been a large area of interest in cancer research. Transcription factors are involved with initiation and regulation of gene transcription; thus, their dysregulation makes them attractive targets as genetic alterations are the driving force behind tumorigenesis [51, 52]. Two principal transcription factors implicated in PaCa formation and progression are Specificity proteins 1 and 3 (Sp1 and Sp3) [53]. Sp1 and Sp3 are part of the zinc finger family of Specificity protein (Sp) family of transcription factors [54].

They regulate their target gene transcription by directly binding to GC-rich elements and promoter sequences. Sp1 and Sp3 initiate gene transcription, however, only Sp3 is able to act as both a transcriptional activator and repressor [55]. These small proteins are ubiquitously expressed in cells and involved in directing various normal biological processes including cell growth, survival, and differentiation [56]. However, their overexpression has been found in a number of different cancers including: lung, breast, prostate, colon, brain, and pancreatic [57-62]. This overexpression has been implicated in tumor formation and progression, metastasis, and angiogenesis [63, 64]. Because of Sp1's association with aggressive cancer and high stage metastasis, it has even been suggested as a potential biomarker to detect a class of advanced pancreatic ductal adenocarcinoma (the most commonly observed type of PaCa) [65]. One gene of particular interest in PaCa regulated by these Sp proteins is survivin [66].

Survivin is encoded by the BIRC5 gene and the smallest member of the inhibitor of apoptosis protein family [67]. Survivin plays a role in both cell cycle regulation and inhibitor of apoptosis. During metaphase and anaphase in cell cycle, survivin is associated with spindle microtubules and ensuring faithful sister chromatid segregation [68]. Survivin prevents the activation of apoptosis by binding to capasases 9 and 3, thus, interrupting caspase 9 from apoptosome formation and sequential cleavage of effector caspase 3 [69]. Increased expression of survivin is observed during fetal development, but in adult cells its expression is minimal [70]. Sp1 and Sp3 are known regulators of survivin transcription and like Sp1 and Sp3, survivin is found to be overexpressed in a variety of different cancers, including pancreatic [71, 72]. Survivin's overexpression is also considered to be a negative prognostic factor for patient survival [73, 74]. This is mainly due to survivin's contribution to the tumor cell's resistance to radiation and chemotherapy treatment [75]. An effective approach to treating PaCa is to pursue the molecular mechanisms that drive tumorigenesis. For these reasons, we are particularly

interested in Sp1, Sp3, and survivin in this study as they are attractive potential therapeutic targets. By downregulating Sp1 and Sp3, survivin expression could perhaps be modulated as well.

NSAIDS AS ANTI-CANCER AGENTS

There have been an increasing number of studies for the potential use of nonsteroidal anti-inflammatory drugs (NSAIDs) as anti-cancer agents. NSAIDs work by inhibiting cyclooxygenase (COX) enzymes 1 and 2 [76]. These catalyze the formation of prostaglandins, a precursor involved with inflammation, pain, and fever. Inhibiting COX enzyme function hinders prostaglandin formation, thus, NSAIDs are used as anti-inflammatory, analgestic, and anti-pyretic agents [77]. Preclinical studies for NSAIDs have demonstrated its use as chemopreventive and chemotherapeutic drugs [78-80]. These have been shown to contain anti-cancer properties, such as inhibition of cancer cell growth and induction of apoptosis [81]. Another aspect that makes NSAIDs an attractive potential agent is that they also have a lower level of toxicity in comparison to conventional chemotherapeutic drugs [82].

Unfortunately, the lack of clinical evidence has prevented replacement of current standard care with NSAIDs. Other limitations of using NSAIDs in cancer therapy are the issues associated with long-term usage of COX inhibitors. Extended use of COX-1 inhibitors can cause gastrointestinal issues while COX-2 inhibitors increase the incidence of cardiovascular events [83-86]. Therefore, there has been a rising interest in finding NSAIDs that work in mechanisms independent of COX inhibition. Consequently, a study was conducted by one of our collaborators. Human pancreatic ductal adenocarcinoma cells (Panc1) were screened with a number of different NSAIDs or COX-1/2 inhibitors for their effect on Sp proteins (Sp1, Sp3, and Sp4) [87]. Cells were treated with 50 μM and collected at 48 h post-treatment. A western

blot was performed to analyze the protein expression of Sp proteins. There were only three NSAIDs that downregulated Sp1 and Sp3, however, tolfenamic acid was found to be the most effective.

Tolfenamic acid (TA) is a common NSAID sold in Europe as a generic medicine for migraine headaches. TA's commercial name is Clotam. Many studies have been conducted from our laboratory and others that demonstrate TA's anti-neoplastic activity in various cancer models [88-94]. TA has been investigated not only because of its ability to induce cell cycle arrest and apoptosis in cancer cells, but also because of its low level of toxicity in non-malignant cells [95]. TA also contains lower levels of toxicity in comparison to other NSAIDs. TA has been shown to work by downregulation Sp1, Sp3, and survivin in PaCa models [53, 96]. A study conducted by our laboratory even used a mouse model to demonstrate that TA treatment sensitizes PaCa cells to radiation by inhibiting survivin expression [97]. For these reasons, TA has been a drug of interest in cancer research to potentially use in combination therapies [98-100].

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CHAPTER – II

RESEARCH APPROACH

TOLFENAMIC ACID AND COPPER(II)-TOLFENAMIC ACID

Thus far, the results seen with TA treatment in PaCa models appear promising. The anti-tumoral properties seen in PaCa cells along with its limited toxicity to normal cells makes TA an attractive anti-cancer agent. It has even been approved for Phase I clinical trials to use in combination therapy with gemcitabine in advanced and/or metastatic PaCa patients. The dosage to induce anti-cancer properties in multiple pre-clinical screening studies is about 50 μM [1-5]. The most practical clinical application could be using this agent in combination with chemotherapeutic agents in clinical testing. Therefore, it is useful to explore the possibility to enhance TA's therapeutic activity to inhibit PaCa cell growth with lower doses.

APPROACH FOR CURRENT STUDY AND HYPOTHESIS

The concept of metallodrugs has been and continues to be an active field in cancer research for their potential use as anti-tumoral agents [6-9]. These are drug derivatives that are modified to contain a metal complex. Metal complexes in metallodrugs have been found to have a synergistic activity with the drug and optimizes the parent drug's bioactivity [10]. Metallodrugs have been proposed to use in combination therapy with standard care in an attempt to sensitize cancer cells that have developed resistance to treatment [11-14]. Their usefulness in combination treatments is due to their anti-cancer properties and limited toxicity [15-17]. In particular, NSAIDs with copper(II) complexes have been investigated in preclinical studies for cancer and reported to have enhanced activity compared to the parent as well as a decreased

toxic effect on gastrointestinal tissues [18]. Interestingly, a novel copper(II) and TA compound has been synthesized and stated to have a higher antioxidant activity in comparison to TA [19]. As literature suggests, the preparation of TA as a copper compound (Cu-TA) could potentially increase its anti-cancer effects. Thus, we hypothesize that Cu-TA can result in enhanced anti-cancer properties and underlying mechanisms in PaCa cells when compared to TA. The ultimate goal in investigating Cu-TA would be to use alongside current standard treatment for PaCa in an attempt to sensitize cancer cells and allow the patients to receive less cytotoxic therapy dosages. Therefore, increasing the overall treatment's activity and decreasing toxicity. The aim of this project is to enhance TA's activity using the Cu-TA derivative and to investigate its anti-cancer properties in PaCa cells.

SPECIFIC AIMS

Specific Aim 1

Characterization of Cu-TA and determining its stability and anti-proliferative activity in human PaCa cell lines.

The physical characterization and chemical stability of Cu-TA compound was first examined by UV-visible spectroscopy and Fourier-transform infrared spectroscopy (FTIR). Then, MIA PaCa-2 and Panc1 cells lines were treated with increasing concentrations of TA or Cu-TA and cell viability was measured 24 and 48 h after treatment. Dose-response curves were plotted and the IC_{50} values were calculated. All further experiments were conducted with the IC_{50} dose for Cu-TA and equimolar TA. Additionally, the biological stability was also tested with compounds that were six-months and one-year old. Since certain NSAIDs are known to cause cardiotoxicity, the cytotoxic effect of Cu-TA was also evaluated using cardiomyocytes, H9C2.

Specific Aim 2

Evaluate the effect of Cu-TA on potential markers associated with PaCa cell growth.

Studies from our laboratory and others have previously demonstrated that TA modulates the expression of Sp1, Sp3 and survivin in PaCa cells. Downregulation of these targets inhibits cell proliferation. TA is also known to induce apoptosis and cell cycle arrest. Western blot and qPCR analysis were performed to examine the effect of Cu-TA on Sp1, Sp3 and survivin protein and mRNA level expression. In addition, modulation of apoptotic markers and cell cycle phase distribution was also analyzed.

Specific Aim 3

Gene expression analysis to precisely elucidate the underlying mechanisms of Cu-TA's anti-cancer activity in PaCa cells.

Gene expression analysis was conducted to elucidate the specific underlying mechanisms of Cu-TA's anti-cancer activity in PaCa cells. Previously we published a molecular profiling delineating the pathway analysis identifying the altered gene expression and associated pathways induced by TA in PaCa cell lines. RNA profiling of Cu-TA treated cells was performed using HTG EdgeSeq Oncology Biomarker Panel. This gene sequencing assay examined 2,560 tumor markers. Pathway analysis was done to demonstrate the functional significance of the altered gene expression. These results were compared to our published work on TA to see if Cu-TA is working in a similar mechanism. It also helped us to identify other underlying mechanisms. After identifying the major regulators, Western blot analysis was performed to confirm these results. Then, key regulators involved with affected molecular and cellular functions were confirmed by qPCR assay using specific primers.

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CHAPTER – III

CHARACTERIZATION OF CU-TA AND DETERMINING ITS STABILITY AND ANTI-PROLIFERATIVE ACTIVITY OF CU-TA IN HUMAN PACA CELL LINES

INTRODUCTION

Numerous studies from our laboratory and others have demonstrated TA's anti-proliferative effect in PaCa models [1-5]. This specific aim is to evaluate Cu-TA's growth inhibitory effect in PaCa cells using a cell viability assay. The results from the assay were used to calculate the IC_{50} value and determine the optimal dosage. Because previous studies have used 24 and 48 h as the time points to investigate TA's anti-cancer properties, we also used these time points. For all subsequent experiments, the IC_{50} value for Cu-TA and equimolar TA were used. Since there have been many studies investigating TA's anti-cancer properties using TA's optimal dosage, equimolar TA rather than TA's IC_{50} value was used to demonstrate Cu-TA's enhanced activity. Physical characterization was performed using Fourier-transform infrared spectroscopy (FTIR) and UV visible spectroscopy to determine Cu-TA's stability as a compound. The biological stability of Cu-TA was also studied by repeating dose curves using a six-month old stock solution and a freshly made stock solution using one year old Cu-TA compound. Another cell viability assay was done using the components used in the synthesis of Cu-TA, $CuCl_2$ and BPY. This was to ensure that the cytotoxic effects we were observing in PaCa cells was due to the Cu-TA compound as a whole and not any single agent. Finally, to test the effects of Cu-TA on non-malignant cells, another cell viability assay was done using cardiomyocyte cells (H9C2).

MATERIALS AND METHODS

Cell Culture:

Two human pancreatic ductal adenocarcinoma cell lines, MIA PaCa-2 and Panc1, were obtained from American Type Culture Collection (Manassas, VA). Both cell lines were grown and cultivated in Dulbecco's Modified Eagle Medium media with high glucose (4500 mg/L) supplemented with fetal bovine serum and penicillin streptomycin as described before [6, 7]. Cells were maintained in an incubator at 37°C with 5% CO_2.

Preparation of Stock Solutions:

Tolfenamic acid was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Copper(II)-tolfenamic acid (Cu-TA) was synthesized by our collaborator Dr. Alvin Holder (Old Dominion University, Norfolk, VA) following the procedure described earlier [8]. The chemical structure of Cu-TA is shown in the appendix. Stock solutions were prepared in dimethyl sulfoxide (DMSO) with a final concentration of 10 mM. $CuCl_2$ and BPY (copper(II) chloride and 2,2′-bipyridine) were the components used to synthesize Cu-TA. Equimolar (representing the concentration of each component in Cu-TA compound) solutions for these components were also prepared in DMSO.

Characterization of Cu-TA complex:

Characterization of Cu-TA was performed using Fourier-transform infrared (FTIR) and UV-visible spectroscopies. FTIR spectroscopy was done using a Bruker Platinum ATR-IR spectrometer, and the UV-visible spectrum was acquired using an Agilent HP8453 diode array spectrophotometer [9]. FTIR and UV spectra analysis was performed at the laboratory of our collaborator, Alvin A. Holder. Initial FTIR and UV spectra were obtained using freshly

prepared Cu-TA powder and these experiments were repeated 8 months later using the same powder.

Cell Viability:

Dose curves to calculate optimal dosages for Cu-TA: MIA PaCa-2 and Panc1 cells were seeded in 96-well plates with each well containing 4,000 cells in 50 µl of media. 10 mM stock concentrations of each drug were diluted in 50 µl of media before treatment. Cells were treated with vehicle (DMSO) or increasing concentrations of Cu-TA (10/25/50 µM) or TA (10/25/50/100 µM). Each treatment was done in triplicates. Cells were then incubated with CellTiter-Glo reagent (Promega, Madison WI) 24 and 48 h post-treatment and incubated in the dark for 15 min. Cell viability was then measured by detecting luminescence using Synergy HT (Bio Tek, Winooski, VT) plate reader. For all subsequent experiments the concentration of Cu-TA's IC_{50} value and equimolar TA were used.

Determining the cytotoxicity of components ($CuCl_2$ and BPY) used for the synthesis of Cu-TA: MIA PaCa-2 and Panc1 cells were treated with DMSO or $CuCl_2$ or BPY using equimolar concentration of Cu-TA's IC_{50} value (MIA PaCa-2: 29 µM; Panc1: 27 µM). Cell viability was measured 24 and 48 h post-treatment.

Cytotoxicity of Cu-TA against non-malignant cells: Cardiomyocytes (H9C2) were treated with increasing dosage of Cu-TA or TA up to equimolar concentration of Cu-TA's IC_{50} value (30 µM). Cell viability was measured post 24 and 48 h using the same method as mentioned previously.

Biological Stability (activity) of Cu-TA: Biological activity testing of Cu-TA was performed using the 6-month-old and one-year-old Cu-TA using pancreatic cancer cells, Panc1. Dose curves were repeated using 12-month-old Cu-TA powder and 6-month-old solution. The dose-response curves were plotted.

RESULTS

Characterization of Cu-TA was performed (Figure 1) to demonstrate Cu-TA's structural stability of Cu-TA complex. Using the FTIR spectrum, the two important stretching frequencies were observed at 1579 cm^{-1} and 1385 cm^{-1} for CO_2(asym) and CO_2(sym), respectively for the coordinated carboxlate anion of TA. In the UV-visible spectrum, the λ_{max} was observed at 680 nm, which was assigned as a d-d transition due to the presence of the Cu(II) metal center. Repeated measurements using 8-month old Cu-TA exhibited very similar spectra, confirming the stability of the compound in powder form. In the freshly prepared powder's FTIR spectrum, the stretching frequencies were observed at 1579 cm^{-1} and 1385 cm^{-1} for CO_2(asym) and CO_2(sym), respectively, while in the 8-month-old powder, the stretching frequencies were observed at 1578 cm^{-1} and 1385 cm^{-1}. The UV-visible spectrum (λ_{ma} at 680 nm) of the fresh and 8-month-old compound (in powder form) exhibited molar extinction coefficient values of 92 M^{-1} cm^{-1} and 89 M^{-1} cm^{-1}, respectively. Additionally, both TA and Cu-TA had a similar effect on cell viability for both cell lines. There was a dose- and time-dependent decrease in cell viability. However, Cu-TA was found to be more effective than TA for inhibiting PaCa cell growth and at lower doses (Table 1 & Figure 2). The IC_{50} values were calculated using the 48 h data and Cu-TA (MIA PaCa-2: 29.32 μM; Panc1: 26.65 μM) was found to be half that of TA's IC_{50} value (MIA PaCa-2: 57.20 μM; Panc1: 61.76 μM). For all subsequent experiments, Cu-TA's IC_{50} value and equimolar TA were the dosages used to treat cells (MIA PaCa-2: 29 μM; Panc1: 27 μM). The biological stability of Cu-TA was then

investigated. Dose curves were repeated using 1) 6-month old stock solution and 2) a freshly made stock solution using one-year old compound. The dose-response and IC_{50} values were compared and found to be consistent for all samples (Figure 3). Both repeated dose curves gave an IC_{50} value within 5% of the original. The cytotoxic effect of the components used in the synthesis of Cu-TA were then tested. Neither $CuCl_2$ and BPY had any significant effect on cell growth in either cell line for both 24 and 48 h post-treatment (Figure 4). Finally, potential cardiotoxicity of Cu-TA was assessed using cardiomyocyte H9C2 cells. Cell viability results demonstrate that neither Cu-TA nor TA caused any significant cytotoxicity to H9C2 cells (Figure 6).

DISCUSSION

The small molecule TA has been demonstrated to contain anti-cancer properties in a myriad of preclinical studies using cancer models. TA's growth inhibitory effect followed a dose- and time-dependent response in PaCa cells [10]. TA has been found to be effective against PaCa cell growth while having a minimal effect on non-malignant cells [11]. Although TA has given promising results as a potential agent to use alongside standard care for PaCa, its high dosage poses an issue. Thus, we began investigating a complex (metallodrug), Cu-TA, in an effort to increase TA's activity.

First, a dose curve was performed to assess the anti-proliferative effect of Cu-TA and calculate the optimal dosage. Both cell lines (MIA PaCa-2 and Panc1) had similar results, TA and Cu-TA caused a decrease in cell viability that was dose-dependent. However, Cu-TA had an IC_{50} value that was half that of TA's. This demonstrates that Cu-TA has an enhanced anti-proliferative activity compared to TA. Having an enhanced growth inhibitory effect means that a lesser dosage of Cu-TA is needed to see the same effect as seen with TA. The characterization

of the compound was performed initially and again after 8 months, and both results were found to be consistent as evaluated by their FTIR and UV visible spectra. The biological (activity) stability was also investigated. Repeated dose curves were done using six-month old stock solution and one-year old compound to calculate their IC_{50} values. The fact that the IC_{50} values were similar to the original meant that the compound was biologically stable and had the same effect on cell viability. Importantly, the anti-cancer activity of Cu-TA in its powder form was retained through 12-month after synthesis. Therefore, the Cu-TA complex is physically and biologically stable for at least a year. The components used in the synthesis of Cu-TA, $CuCl_2$ and BPY, were also evaluated for their effect on cell viability. There was no effect on cell viability when using equimolar Cu-TA's IC_{50} value at 24 or 48 h post-treatment. This establishes that the growth inhibitory response seen in the Cu-TA treatment is due to the Cu-TA compound as a whole and not any single agent acting alone. Furthermore, while an anti-proliferative effect is desired in cancer cells, the drug of interest should not have an effect in non-malignant cells. When investigating a potential drug in cancer research, the toxicity to normal cells is just as important as effectiveness of the drug. In this study, cardiomyocytes (H9C2) were used as the non-malignant cells. Certain NSAIDs are known to cause cardiotoxicity [12-15]. Thus, since Cu-TA is a NSAID derivative it is important to determine its cytotoxic effect in H9C2 cells. Cu-TA had no significant effect on cardiomyocyte cell viability.

In conclusion, we were able to determine the optimal dosages of Cu-TA that will be used in the next experiments. The enhanced activity of Cu-TA meets one of the purposes of our study, to increase TA's activity so that less dosage is required to see its anti-cancer effects. FTIR/UV-visible spectroscopy and repeated dose curves demonstrate the physical and biological stability of Cu-TA, respectively. Finally, the fact that Cu-TA does not have a significant effect on

cardiomyocyte cell viability meets another purpose of our study, to investigate potential agents that are effective and have minimal toxicity.

FIGURES

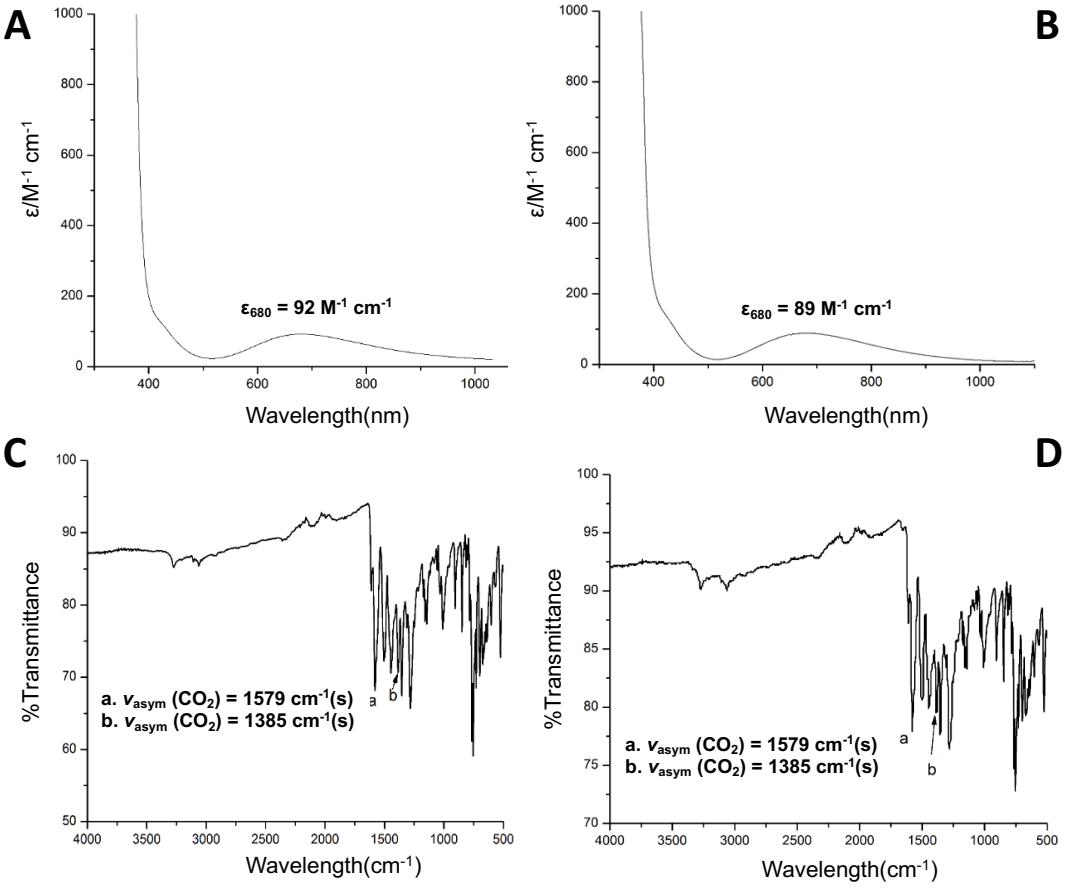

Figure 1. Physical Characterization of Cu-TA: Physical characterization and stability of Cu-TA was performed using (A&B) UV-visible spectroscopy and (C&D) FTIR spectroscopy. Tests were performed with (A&C) freshly prepared compound and (B&D) 8-month old compound stored at room temperature.

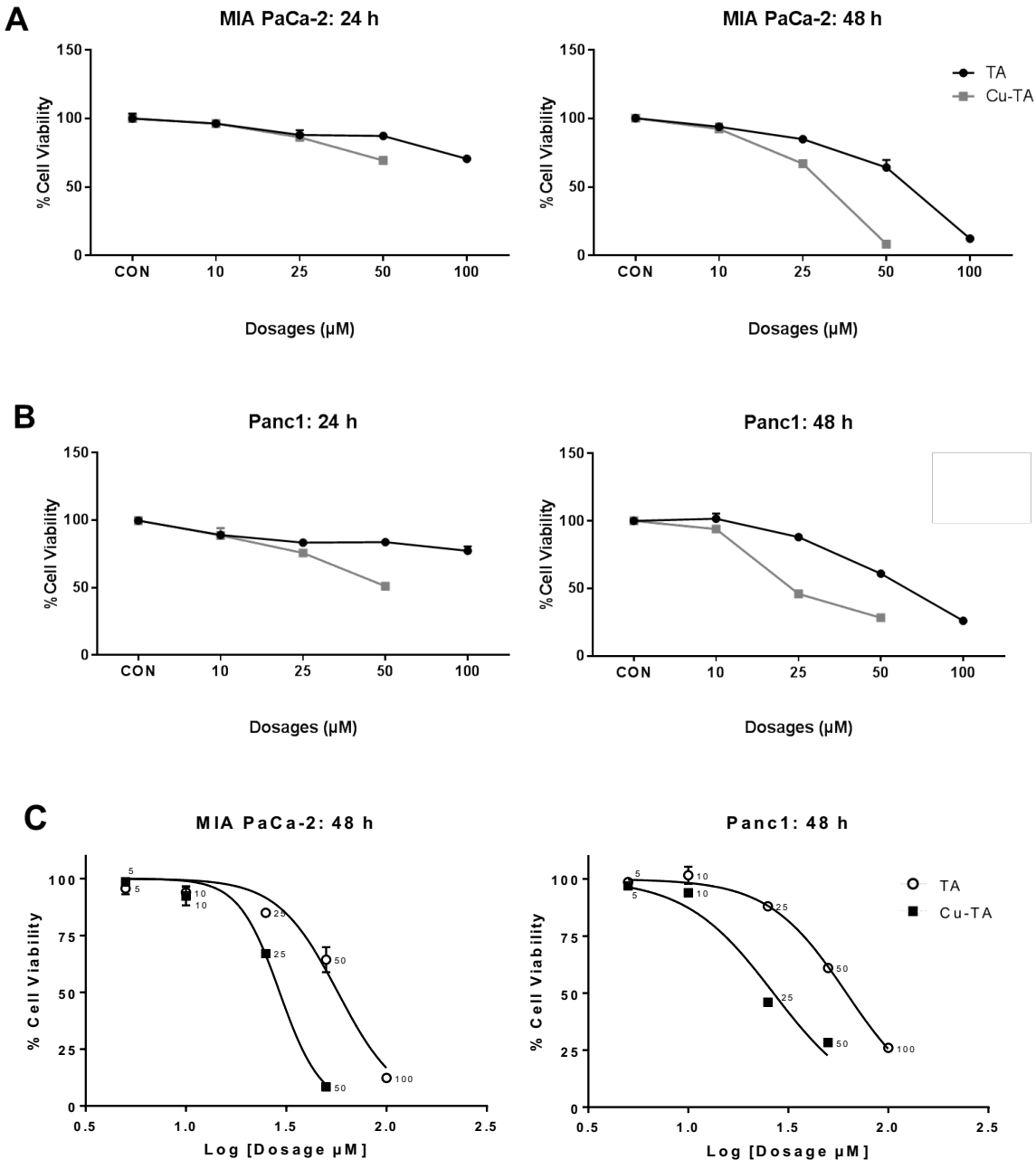

Figure 2. Anti-proliferative effect of TA or Cu-TA against PaCa cells: (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO (vehicle) or TA (10–100 µM) or Cu-TA (10–50 µM) and cell viability was measured after 24 and 48 h. (C) dose curves for 48 h data for both MIA PaCa-2 and Panc1 cells using log values to calculate the IC_{50} values. Bars represent % viable cells (mean ± SD) normalized to control. All treatments were done in triplicate.

Cell line	IC_{50} Value TA	IC_{50} Value Cu-TA
MIA PaCa-2	57.20 µM	29.32 µM
Panc1	61.76 µM	26.65 µM

Table 1: IC_{50} Values for PaCa cell lines at 48 h post-treatment with TA or Cu-TA.

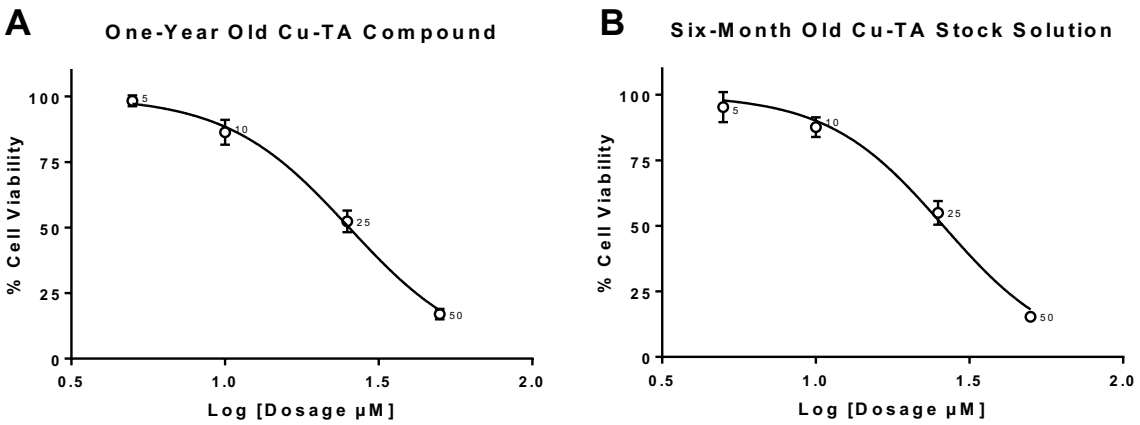

Figure 3. Stable biological activity of Cu-TA: Panc1 cells were treated with increasing dosages of (A) one-year old Cu-TA compound and (B) six-month old stock solution of Cu-TA. The IC_{50} values were calculated using GraphPad Prism V6.0. All treatments were done in triplicate. All treatments were done in triplicate, and the bars marked with '*' are significantly different from corresponding control ($p < 0.05$).

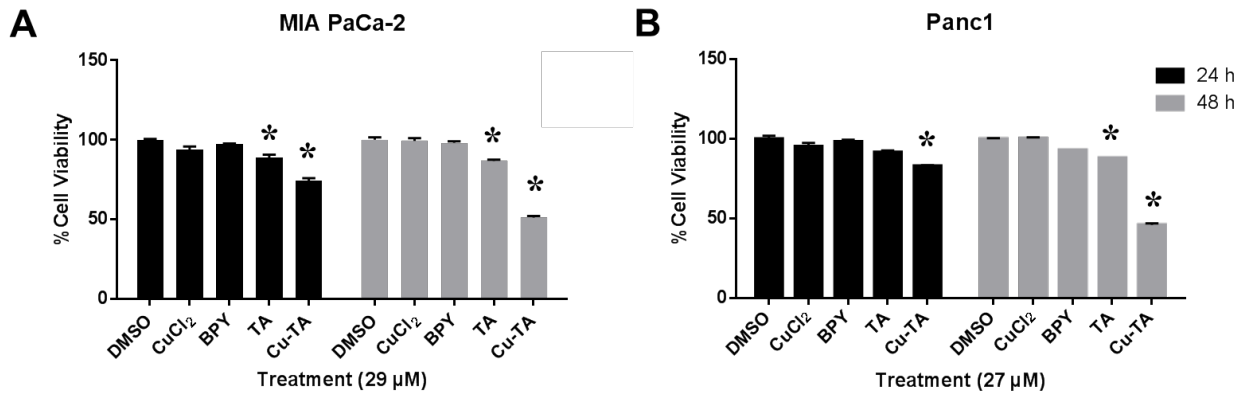

Figure 4. Cu-TA components, $CuCl_2$ and BPY, on PaCa cell growth: The effect of $CuCl_2$ and BPY on cell viability in (A) MIA PaCa-2 and (B) Panc1 (B) cells was measured 24 and 48 h post-treatment. Bars represent % viable cells (mean ± SD) normalized to control. All treatments were done in triplicate, and the bars marked with '*' are significantly different from corresponding control ($p < 0.0001$).

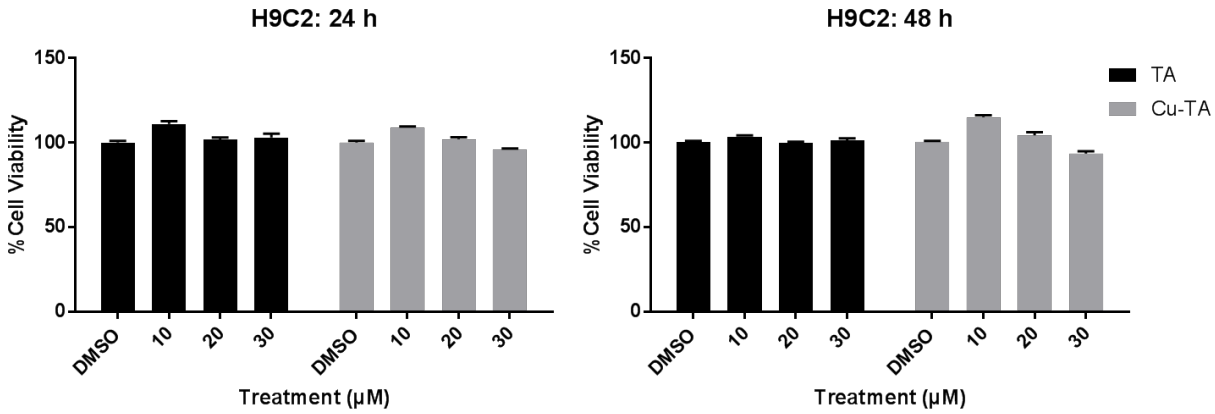

Figure 5. Cytotoxicity of Cu-TA in cardiomyocytes: To determine the effect of Cu-TA on non-malignant cells, we have evaluated the cardiotoxicity using cardiomyocytes, H9C2. Cells were treated with increasing dosages of Cu-TA or TA (10, 20, 30 µM) and cell viability was measured at 24 and 48 h. All treatments were done in triplicate.

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CHAPTER – IV

EVALUATE THE EFFECT OF CU-TA ON POTENTIAL MARKERS ASSOCIATED WITH PACA CELL GROWTH

INTRODUCTION

Over the last few decades, the cancer research community has grown immensely. Unfortunately, pancreatic cancer (PaCa) is one of the few cancers that has not made a significant improvement in terms of treatment and survival rates. In the U.S., PaCa is the 3rd leading cause of cancer related deaths and is one of the most fatal malignancies due to its poor prognosis [1-3]. PaCa diagnosis generally occurs in the late stages, after the cancer has metastasized and is aggressive in nature. There are currently no early detection methods for this cancer; which contributes to its late diagnosis and thus treatment is quite difficult [4]. Current standard treatment for PaCa include surgery and chemotherapy along with radiation. However, because of its late diagnosis, surgery is a viable option to less than 20% of cases [5]. Chemotherapy drugs for PaCa include gemcitabine, 5-fluorouracil, cisplatin, and paclitaxel [6, 7]. These cytotoxic therapies have a number of negative side effects including low blood count and peripheral neuropathy [8]. Certain chemotherapy drugs have additional side effects. For example, at higher dosages cisplatin can cause nerve and renal damage [9]. Notably, chemotherapy and radiation becomes less effective when the patient begins developing resistance over time [10, 11]. This is a serious concern, since neither dosage can be increased because of the morbidity associated with chemotherapy and irradiation. Thus, there is an urgent need for alternative therapies that are more effective yet less harmful.

Survivin is encoded by the BIRC5 gene and part of the inhibitor of apoptosis protein family [12]. Increased expression of survivin is observed during fetal development, but in adult cells its expression is minimal [13]. Survivin's overexpression is considered to be a negative prognostic factor for patient survival [14, 15]. Satoh et al., evaluated the clinical specimens of 56 patients and survivin was found to be over-expressed in 77% of pancreatic ductal carcinoma patients [16]. In another study, Lee et al., found that 94% of 49 patient samples were positive to survivin [17]. Furthermore, survivin was shown as a constitutive resistance factor for radiation [18] and knocking down of survivin via siRNA technology attenuated radiation resistance [19]. Since the association of survivin with PaCa and the response to PaCa treatment is well established, it is ideal to exploring the agents that can target survivin and can induce anti-cancer activity.

Tolfenamic acid (TA) is classified as a non-steroidal anti-inflammatory drug (NSAID) and is used as a generic drug to treat migraines in Europe. Studies from our laboratory and others have demonstrated that TA contains anti-cancer properties such as cancer cell growth inhibition and induction of apoptosis in pre-clinical models, including PaCa [20-23]. TA also sensitizes PaCa cells and tumors in mice (orthotopic model) to radiation [24]. TA has been shown to work through downregulation of Specificity proteins 1 and 3 (Sp1 and Sp3) [20]. Sp1 and Sp3 are part of the zinc finger family of Specificity protein (Sp) family of transcription factors [25]. They can regulate their target gene transcription by binding to GC-rich elements and promoter sequences. These small proteins are ubiquitously expressed in cells and involved in directing various normal biological processes including cell growth, survival, and differentiation [26]. However, their overexpression has been found in a number of different cancers including: lung, breast, prostate, colon, brain, and pancreatic [27-32]. This overexpression associated with tumor formation and progression, metastasis, and angiogenesis [33]. Because of Sp1's

association with aggressive cancer and high stage metastasis, it has even been suggested as a potential biomarker to detect a class of advanced pancreatic ductal adenocarcinoma [34].

One gene of particular interest in PaCa regulated by these Sp1 and Sp3 is survivin [35]. For these reasons, TA is an attractive potential chemotherapeutic for PaCa since it targets Sp1, Sp3, and survivin. TA has been approved for Phase I Clinical Trials along with radiation and gemcitabine treatment for PaCa. However, since the dosage for TA's anti-cancer activities is slightly high (50 μM), strategies to enhance its activity is important. Therefore, we began to look into using a TA derivative, specifically, a metallodrug. Metal complexes have been found to have a synergistic activity with their original drugs which enhance the drug's bioactivity [36-42]. Recently, a copper (II)-Tolfenamic acid has been synthesized and published [43].

As demonstrated in the previous chapter, Cu-TA was found to have an anti-proliferative effect on PaCa cells. To see if Cu-TA is affecting these markers associated with PaCa growth, their expression was analyzed. Both protein and mRNA expression levels were examined to see if their downregulation was on a transcriptional or post-translational level. Next, Cu-TA's effect on apoptosis as well as cell cycle phase distribution was investigated since induction of apoptosis and cell cycle arrest can contribute to growth inhibition in cells. Apoptosis was assessed by looking at various apoptotic-associated markers including effector caspase 3/7 activity, cleaved PARP protein expression, and apoptotic cell populations. Cell cycle phase distribution was examined using a cell cycle assay.

MATERIALS AND METHODS

Western blot:

MIA PaCa-2 and Panc1 cells were treated with either DMSO, Cu-TA (MIA PaCa-2: 29 μM; Panc1: 27 μM) or equimolar TA. After 24 and 48 h treatments, cell lysates were collected and prepared. Total cellular protein was extracted using cell lysis buffer and protein quantification was done using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Protein samples were then separated through 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane. Next, the membranes were blocked with 5% milk in Tris-Buffered Saline with 1% Tween. Blots were incubated with primary antibody overnight and incubated with secondary antibody for one hour the following day. Bands were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Protein expression of Sp1 (Santa Cruz Biotechnology, Dallas, TX), Sp3 (Santa Cruz Biotechnology), cleaved PARP (Cell Signaling Technology, Danvers, MA), survivin (R&D Systems, Minneapolis, MN), cyclin B1 (Cell Signaling Technology), and cyclin A (Cell Signaling Technology) were evaluated using specific antibodies while the expression of β-actin (Sigma-Aldrich Corporation) was used as a loading control.

Quantitative Polymerase Chain Reaction (qPCR):

MIA PaCa-2 and Panc1 cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 μM; Panc1: 27 μM) or equimolar TA for 48 h. At the time of collection, total RNA was extracted from treated cells using TRIzol (Invitrogen, Carlsbad, CA). Total RNA was then converted into single-stranded cDNA using Superscript III (Invitrogen). After cDNA amplification, Sp1, Sp3 and survivin were probed for using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and samples were placed in a 96-well LightCycler 96 Real-Time PCR system (Roche) for qPCR analysis. Each sample was done in triplicates and GAPDH was used as a housekeeping gene.

Caspase 3/7 Assay:

MIA PaCa-2 and Panc1 cells were seeded in 96-well plates with each well containing 4,000 cells in 50 µl of media. 10 mM stock concentrations of each drug were diluted in 50 µl of media before treatment, with each treatment done in triplicates. The activation of caspase 3 and 7 was measured using Caspase-Glo 3/7 kit (Promega). Cells were platted in a 96-well plate and then treated with DMSO, Cu-TA (MIA PaCa-2: 29 µM; Panc1: 27 µM) or equimolar TA. At 24 and 48 h, samples were incubated with Caspase 3/7-Glo substrate for 1 h in the dark and then read by Synergy HT (Bio Tek) plate reader.

PE Annexin V Staining:

Apoptotic cell populations were detected using PE Annexin V Apoptosis Detection Kit I (BD Biosciences). 125,000 MIA PaCa-2 and Panc1 cells were seeded in 2 ml of media. The next day, cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 µM; Panc1: 27 µM) or equimolar TA for 24 and 48 h. Samples were collected, washed with PBS, and then incubated with Annexin V-PE/7-AAD solution for 15 min in the dark. Apoptotic cell populations (10,000 cells) were measured via FC500 flow cytometer.

Cell Cycle Arrest:

125,000 MIA PaCa-2 and Panc1 cells were seeded in 2 ml of media in a 6 well plate. The next day cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 µM; Panc1: 27 µM) or equimolar TA then collected at 24 and 48 h post-treatment. Samples were collected, washed with PBS, fixed with cold 70% ethanol and then stored in -80 ℃ overnight. The collected samples were then incubated with propidium iodide(PI)/RNAse solution for DNA staining for 15 min at room temperature in the dark. Finally, samples were then incubated in 37°C for 10 min. The cell cycle phase distribution was analyzed (10,000 cells) using FC500 Flow Cytometer.

RESULTS

The Cu-TA treatment was found to downregulate Sp1, Sp3 and survivin protein expression (Figure 6) at 48 h in both cell lines. TA is also known to downregulate Sp1, Sp3 and survivin protein expression, however, at the equimolar dosage of Cu-TA's IC_{50} value there was no change. Quantification of Western blots are given in Figure 7A&B, while the fold change of survivin protein expression revealed several fold (MIA PaCa-2: 5.2-fold; Panc1: 6.4-fold) higher in Cu-TA compared to TA (Figure 7C). qPCR results demonstrated that TA and Cu-TA treatments significantly lower survivin mRNA expression levels in both cell lines. However, Sp1 and Sp3 mRNA expression levels remained relatively unchanged in both cell lines (Figure 8). Cu-TA treatment increased caspase 3 and 7 activity as well as protein expression of apoptotic marker c-PARP (Figure 9). Cu-TA also caused an increase in apoptotic cell populations (Figure 10A&B). Figure 10C shows the fold change in apoptotic cell populations. The Cu-TA treatment in Panc1 cells revealed a significant increase in G_2/M phase cells compared to the control, indicating cell cycle arrest (Figure 11). Confirmation of G_2/M phase arrest was done by examining protein expression of G_2/M markers, cyclin B1 and cyclin A (Figure 12). There was no cell cycle arrest observed in MIA PaCa-2 cells at 24 or 48 h.

DISCUSSION

The zinc finger transcription factors Sp1 and Sp3 play a major role in the tumor formation and progression in PaCa [25]. These transcription factors regulate genes involved in many biological processes including cell growth and survival, cell cycle, and apoptosis [33]. They also regulate survivin, a gene that plays a role in apoptosis inhibition and cell cycle regulation [35]. Their expression was evaluated for their implication in PaCa growth as well as TA's

known downregulation of these potential markers. Western blot analysis revealed a downregulation of Sp1, Sp3 and survivin at 48 h in the Cu-TA treatment. Their mRNA expression levels were then evaluated at 48 h since protein downregulation was observed at that time point. Survivin was found to be significantly decreased while Sp1 and Sp3 levels were comparatively unchanged. This indicates that Cu-TA effects survivin on a transcriptional level while Sp1 and Sp3 are affected post-translationally. TA is known to downregulate Sp1 and Sp3 post-translationally, through proteasome dependent degradation [44]. This data suggests that Cu-TA could perhaps be working in a similar mechanism.

Next, Cu-TA's induction of apoptosis was assessed. Apoptosis can be induced one of two ways: intrinsic pathway or extrinsic pathway. The intrinsic pathway is characterized by mitochondrial outer membrane permeability and release of cytochrome c. This leads to the formation of apoptosomes and cleavage of initiator caspase 9 [45]. The extrinsic pathway is induced by an appropriate ligand binding to the death receptor on the cell's outer membrane. This activates the death domain and cleaves the initiator caspase 8 [46]. The intrinsic and extrinsic pathway have different initiator caspases, but these pathways converge on the activation of effector caspases 3 and 7. Caspase 3 and 7 then go on to cause DNA fragmentation, membrane blebbing, and damage to house-keeping proteins, ultimately leading to cell death [47, 48]. Effector caspase 3 and 7 are also responsible for cleaving PARP1 (a DNA repair protein) [49]. The caspase 3/7 assay examined caspase 3 and 7 activity and revealed a significant increase in the Cu-TA treatment. This coupled with the protein expression of c-PARP indicated the activation of apoptosis. Sp1, Sp3 and survivin all have a role in apoptosis. Specifically, survivin inhibits caspase 9 and 3 cleavage [50]. Perhaps by inhibition of survivin, caspase cleavage was able to occur and induce apoptosis. The detection of apoptotic cell populations was also analyzed. Apoptosis is characterized by several different

features, including membrane asymmetry. Phosphatidylserine (PS) is located on the cell's inner membrane but becomes translocated to the outer membrane during the early events of apoptosis [51]. Annexin V is a protein that has a high affinity for PS and can be conjugated to a fluorochrome phycoerythrin (PE) to detect early apoptotic cells using flow cytometry. The PE Annexin V Detection Kit used to analyze apoptotic cell populations was also done with another dye, 7-Amino-Actinomyocin (7-AAD). Cells that are viable are impermeable to 7-AAD and cells that are dead, either due to necrosis or apoptosis, are able to take up this dye. Membrane asymmetry occurs during early apoptosis but will remain during late apoptosis and/or necrosis. Since this assay does not distinguish whether cells have died as a result of necrosis or apoptosis, it is necessary to stain for both dyes. If cell populations are visualized as viable, early apoptosis, and late apoptosis/necrotic, then this implies that cells are progressing through apoptosis. The Cu-TA treatment showed a significant increase in apoptotic cell populations at 48 h for both cell lines. This demonstrates the occurrence of apoptosis.

Then, cell cycle phase distribution was analyzed using the Cell Cycle Detection Kit. This assay uses propidium iodide (PI) staining to see the cell cycle stage a cell is in. PI is a DNA intercalating dye that is fluorescent. Thus, the fluorescent intensity is proportional to the amount of DNA present. In G_1 phase, cells are increasing their size and preparing to replicate DNA. In S phase the cells begin replicating their DNA, thus they have more DNA than G_1 phase [52]. In G_2 phase the cells will have completed DNA replication, thus their fluorescent intensity will be twice as much as G_1 phase [53]. In Panc1 cells, there was a significant increase in G_2/M phase with the Cu-TA treatment at 48 h. This accumulation of cells suggests that G_2/M phase arrest is occurring. To confirm these cell cycle results, a western blot was done at 48 h to analyze protein expression of G_2/M phase cyclin markers. Cyclins are proteins that play a role in the progression of cell cycle phases by forming complexes with cyclin-dependent

kinases (CDKs) [54]. Cyclins regulate CDK activity by their differential expression; cyclins are expressed in the phase they are needed and subsequently degraded afterwards. These complexes have various activities including regulation of target gene transcription, however, their exact function depends on the specific complex and the cell cycle phase they are in [55]. Expression of cyclin B1 and A were examined because of their known role in G_2/M phase [56, 57]. Cyclin A has the highest expression, meaning highest activity, in G_2 phase while cyclin B1 has its peak expression in M phase [58]. Expression of both cyclins is required to progress through the G_2/M transition. Protein expression of both cyclins were inhibited with the Cu-TA treatment. This corroborates cell cycle results that Cu-TA causes G_2/M arrest. Survivin is known to play a role in cell cycle regulation. Survivin is expressed during the M phase of cell cycle and associates/stabilizes the microtubules of the mitotic spindle [59, 60]. The Cu-TA-induced downregulation of survivin may be partially responsible for the G_2/M phase arrest that is occurring in Panc1 cells. Since survivin is being inhibited, the spindle apparatus stability is not reinforced and thus cell cycle arrest may occur. There was no observed cell cycle arrest in MIA PaCa-2 cells, suggesting that Cu-TA is predominately affecting apoptotic pathways in MIA PaCa-2 cells.

To summarize, Cu-TA causes a growth inhibitory effect in PaCa cells. This anti-proliferative effect was partially due to induction of apoptosis and cell cycle arrest. The activation of apoptosis was demonstrated by an increase in effector caspase activity, c-PARP expression, and apoptotic cell populations. Cell cycle arrest was observed at G_2/M phase in Panc1 cells and these findings were confirmed by analyzing protein expression of associated markers. Additionally, molecular regulators that drive tumorigenesis and resistance, Sp1 and Sp3, were downregulated with Cu-TA. Expression of one of their targets, survivin, was also found to be significantly decreased. While we see a trend in inhibition of Sp1 and Sp3, the most responsive

marker is survivin, with MIA PaCa-2 having 82.8% protein inhibition and Panc1 86.7%. This further supports that the anti-cancer activity seen with Cu-TA may be more associated with survivin. In the TA treatment, Sp1, Sp3 and survivin protein expression are all relatively unaffected. However, in the Cu-TA treatment, the effect is higher in survivin when compared to Sp1 and Sp3. Cu-TA was found to have a significant increase in survivin inhibition (MIA PaCa-2: 5.2-fold; Panc1: 6.4-fold) compared to TA. Both Sp1 and Sp3 regulate survivin and can inhibit or activate its expression. If the downregulation of survivin is through Sp proteins inhibition (post-translational degradation), then the effect observed is an additive response.

In conclusion, survivin is a tangible marker to target for treating cancers due to its strong association with tumorigenesis and resistance to therapy and the specific agents to target survivin are still under investigation. Cu-TA showed anti-cancer activity in PaCa cells and had an enhanced activity in comparison to TA via inhibiting survivin. Finding a potent inhibitor of survivin for PaCa treatment is just as vital as finding a non-toxic and stable agent. The ultimate goal of investigating Cu-TA would be to use this alongside standard treatment care. By downregulating survivin, we hope to sensitize the cancer cells to treatment.

FIGURES

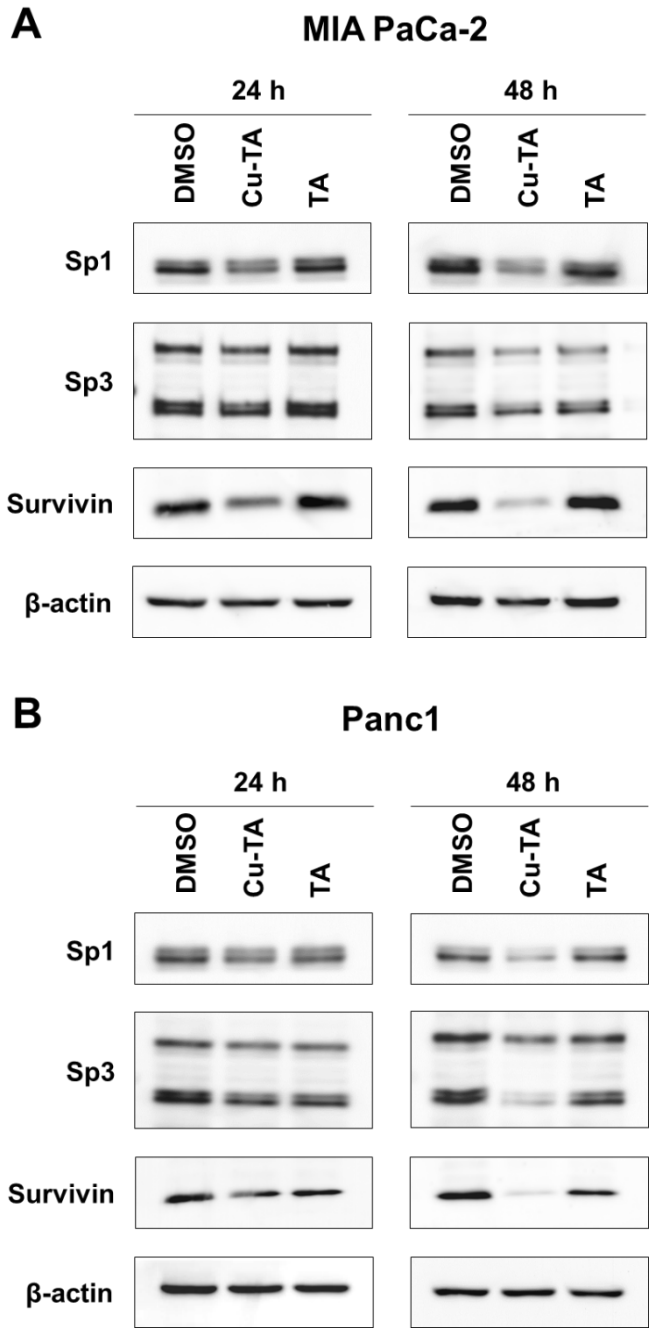


Figure 6: Cu-TA inhibits protein expression of Sp1, Sp3 and survivin. (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO, Cu-TA or TA for 24 and 48 h. Protein extracts from cell lysates were then prepared and the expression of Sp1, Sp3, survivin and β-actin was determined by Western blot analysis.

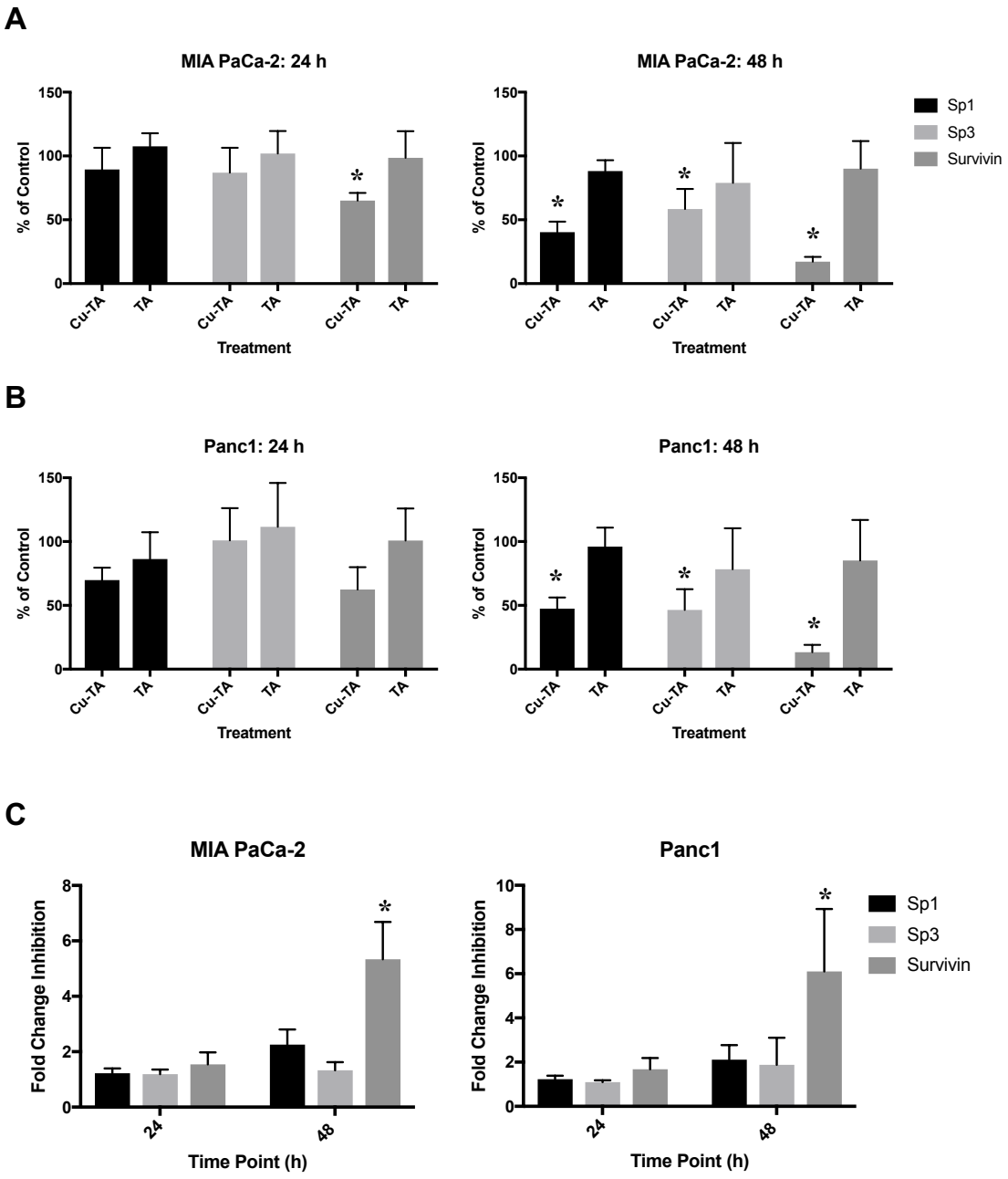


Figure 7: Quantification of Western blot results for Sp proteins and survivin. Western blot quantification of Sp1, Sp3 and survivin protein expression in (A) MIA PaCa-2 and (B) Panc1 cells was determined at 24 and 48 h post-treatment. Bars represent % protein expression (mean ± SD) normalized to control. (C) The fold change of Sp1, Sp3 and survivin inhibition in Cu-TA compared to TA was calculated from the quantification results in both cell lines at 24 and

48 h. All treatments were done in triplicate, and the bars marked with '*' are significantly different from corresponding control ($p < 0.05$).

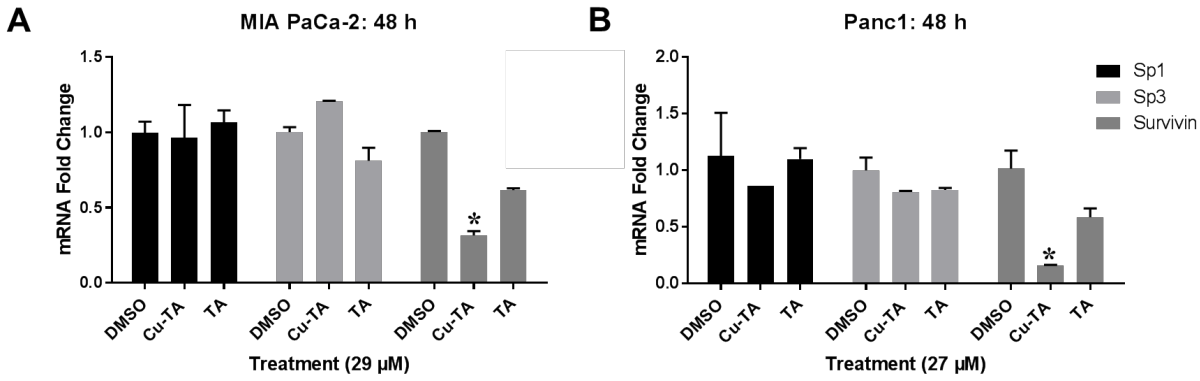


Figure 8: Cu-TA effects survivin on a transcriptional level. (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO, TA or Cu-TA for 48 h. Total RNA was then extracted and mRNA expression levels of Sp1, Sp3 and survivin were assessed via qPCR. GAPDH was used as a housekeeping gene. Bars represent mRNA fold change (mean ± SD) normalized to control. All treatments were done in triplicates, and the bars marked with '*' are significantly different from corresponding control ($p < 0.0001$).

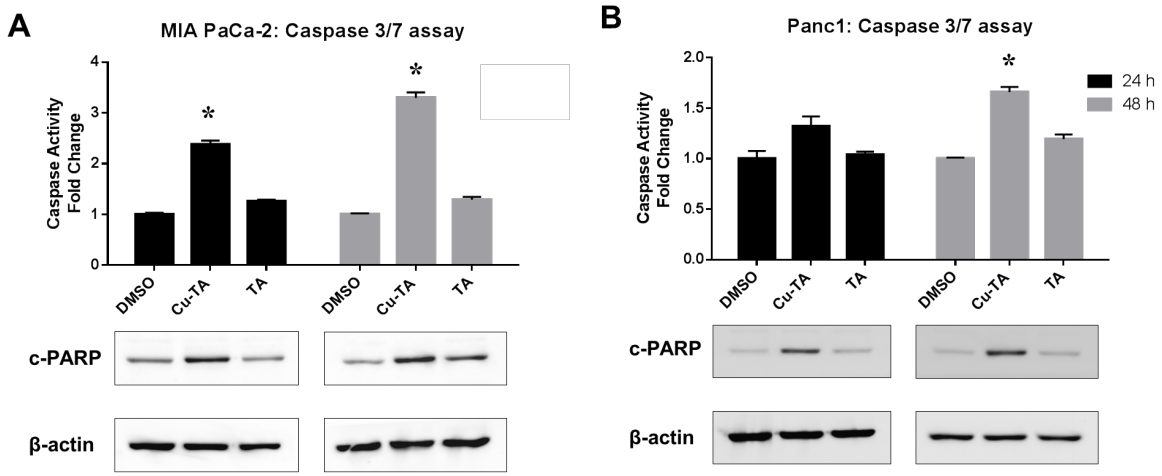
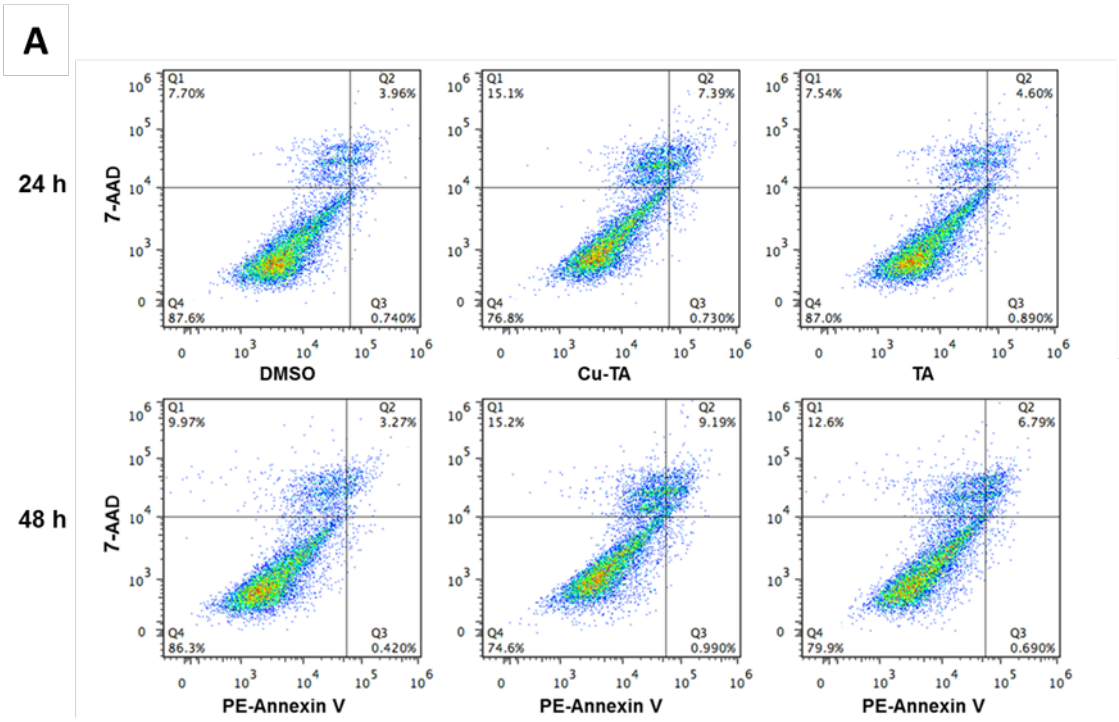


Figure 9: Cu-TA increases caspase activity and c-PARP expression. (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO or IC_{50} value of Cu-TA (MIA PaCa2: 29 µM; Panc1: 27 µM) or equimolar TA. Caspase 3/7 activity and c-PARP protein expression was determined at 24 and 48 h post-treatment. Bars represent mean ± SD. All treatments were done in triplicates, and the bars marked with '*' are significantly different from corresponding control ($p < 0.0001$).



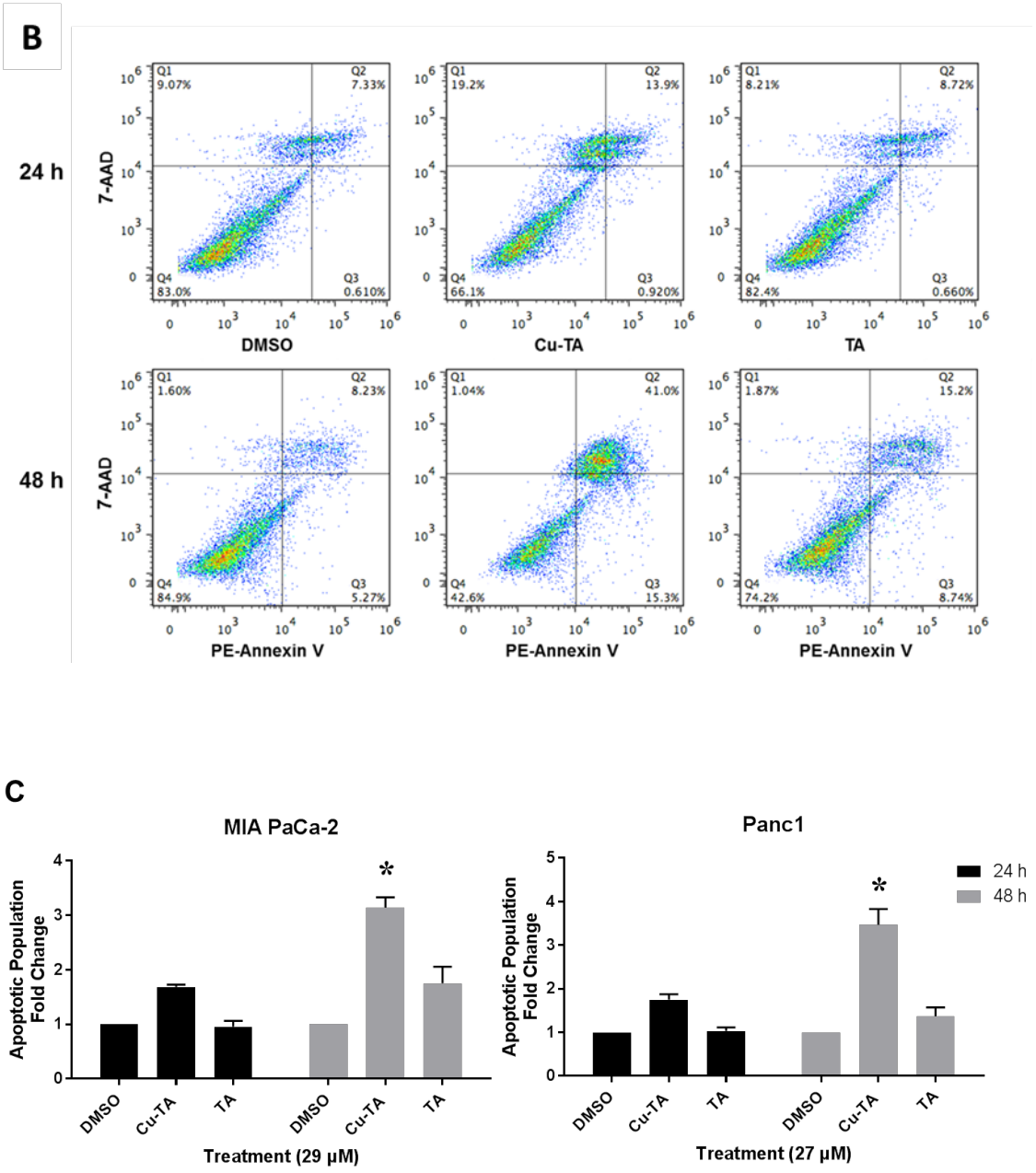


Figure 10: Cu-TA increases apoptotic cell populations. (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO, Cu-TA or TA. Apoptotic cell populations were assessed using PE Annexin V Staining post 24 and 48 h treatment and (C) the fold change in apoptotic cell populations was calculated. Bars represent mean ± SD. All treatments were done in triplicates, and the bars marked with '*' are significantly different from corresponding control ($p < 0.0001$).

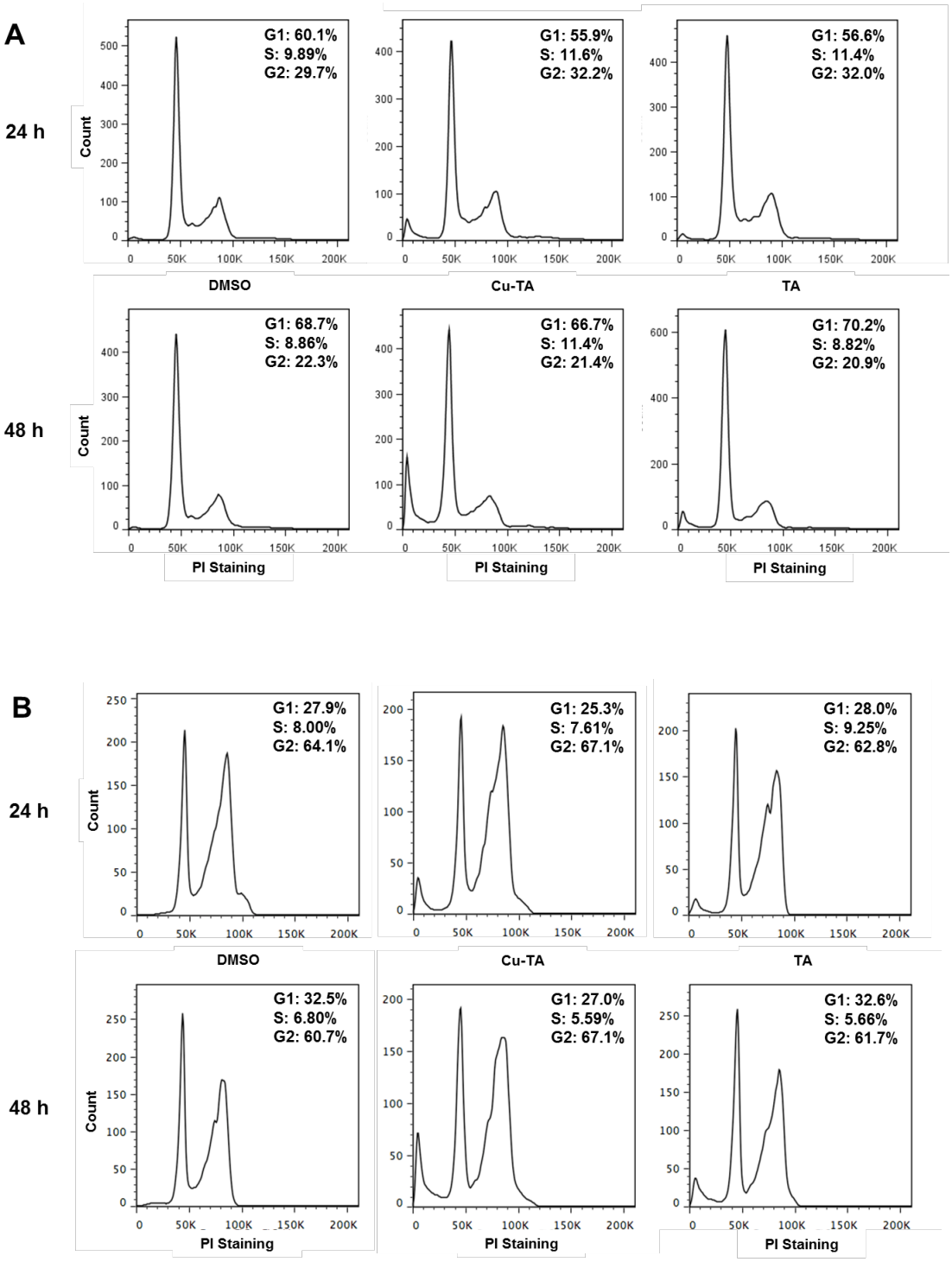


Figure 11: Cu-TA causes arrest at G_2/M phase in Panc1 cells. (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO, Cu-TA or equimolar TA for 24 and 48 h post-treatment. Cells were collected and then incubated with propidium iodide DNA staining. Samples were

then analyzed via flow cytometry. Quantitative representation of Panc1 48 h results (C) shows the experiments done in triplicates, and the bars marked with '*' are significantly different from corresponding control ($p < 0.05$).

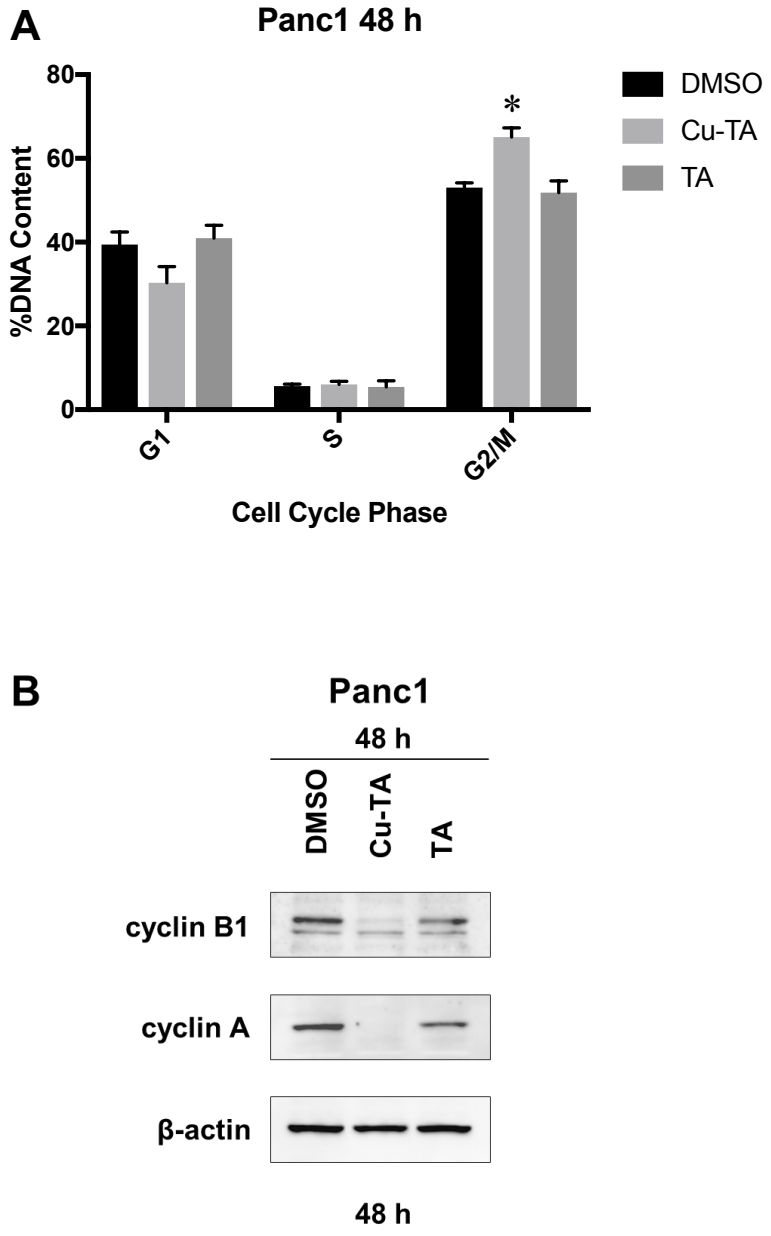


Figure 12: Confirmation of G_2/M phase arrest in Panc1 cells. Panc1 cells were treated with DMSO or Cu-TA (27 μM) or equimolar TA for 48 h. (A) Cells were collected and then incubated with propidium iodide DNA staining. Samples were then analyzed via flow

cytometry. Bars represent % cells in a particular cell cycle phase (mean ± SD) normalized to control. All treatments were done in triplicate, and the bars marked with '*' are significantly different from corresponding control ($p < 0.05$). (B) Protein extracts from cell lysates were then prepared and the expression of G_2/M phase markers cyclin B1, cyclin A and β-actin was determined by Western blot analysis.

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CHAPTER – V

GENE EXPRESSION ANALYSIS TO PRECISELY ELUCIDATE THE UNDERLYING MECHANISMS OF CU-TA'S ANTI-CANCER ACTIVITY IN PACA CELLS

INTRODUCTION

While many types of cancers have made considerable improvements in their overall survival rate and advancements in treatment, pancreatic cancer (PaCa) is one of the few that continues to be a fatal malignancy [1, 2]. Currently, the overall five-year survival rate is dismal (~8%) [3]. Approved treatment options for PaCa are limited in terms of selection and effectiveness. Standard treatment is typically chemotherapy (usually a combination of chemo agents) and radiation. Chemotherapy drugs for PaCa include gemcitabine, 5-fluoruracil, cisplatin, paclitaxel, and erlotinib [4, 5]. These cytotoxic therapies have a number of negative side effects such as vomiting, hair loss, low blood count and peripheral neuropathy [6]. Surgery is another treatment option; however, this approach is not feasible to more than 80% of patients due to late stage diagnosis [7-9]. When patients begin treatment, the cancer is often at an advanced stage and has spread to local organ sites. Another aspect of PaCa that adds to its fatality is the cancer cells' inherent or acquired resistance to treatment on both a physiological and molecular level. The resistance to chemotherapy and radiation poses a serious issue for patients since neither dosage can be increased due to the high level of toxicities [10, 11]. This provides an urgent need for improved alternative treatment options for PaCa that more sensitizing, effective and less toxic.

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) as anti-tumoral agents has gained tremendous popularity. NSAIDs work by inhibiting cyclooxygenase (COX) enzymes 1 and 2 [12]. Preclinical studies for NSAIDs have demonstrated its use as chemopreventive and chemotherapeutic drugs [13-15]. They have been shown to contain anti-cancer properties, such as inhibition of cancer cell growth and induction of apoptosis [16]. Another aspect that makes NSAIDs an attractive potential agent is that they also have a lower level of toxicity in comparison to conventional chemotherapeutic drugs [17]. One NSAID of particular interest is tolfenamic acid (TA). TA is a common NSAID sold in Europe as a generic medicine for migraine headaches. Many studies have been conducted from our laboratory and others that demonstrate TA's anti-neoplastic activity in various cancer models, including PaCa [18-24]. TA has been investigated not only because of its ability to induce cell cycle arrest and apoptosis in cancer cells, but also because of its low toxicity in non-malignant cells [25]. TA also elicits lower levels of toxicity in comparison to other NSAIDs. It has been shown to work by COX-independent mechanisms, by downregulation of Specificity proteins 1 and 3 (Sp1, Sp3) in PaCa models [26, 27]. The zinc finger transcription factors Sp1 and Sp3 play a major role in the tumor formation and progression in PaCa [28]. These transcription factors regulate genes involved in many biological processes including cell growth and survival, cell cycle, and apoptosis [29]. They also regulate survivin, a gene that plays a role in apoptosis inhibition and cell cycle regulation [30]. We even used a mouse model to show that TA treatment sensitizes PaCa cells to radiation treatment by inhibiting survivin expression [31]. For these reasons, TA has been a drug of interest in cancer research to potentially use in combination therapies [32-34].

Thus far, the success seen with TA treatment in PaCa models appear promising. The anti-neoplastic properties seen in PaCa cells along with its limited toxicity to normal cells makes

TA an attractive agent for treating cancer. It has even been approved for Phase I clinical trials to use in combination therapy with gemcitabine in advanced and/or metastatic PaCa patients. Although TA has given promising results as a potential agent to use alongside standard care for PaCa, its high dosage poses an issue. Therefore, we began exploring the options to enhance the anti-neoplastic properties of TA. The concept of metallodrugs has been and continues to be an active field in cancer research [35-38]. Metallodrugs have been proposed to use in combination therapy with standard care in an attempt to sensitize cancer cells that have developed resistance to treatment [39-42]. Their usefulness in combination treatments is due to their anti-cancer properties and limited toxicity [43-45]. In particular, NSAIDs with copper(II) complexes have been investigated in preclinical studies for cancer and reported to have enhanced activity compared to the parent drug, as well as a decreased toxic effect on gastrointestinal tissues [46].

We have demonstrated that TA and copper complex (Cu-TA) results in enhanced anti-proliferative activity in PaCa cells when compared to TA. In this aim, gene expression analysis was conducted to elucidate the specific underlying mechanisms of Cu-TA's anti-cancer activity in PaCa cells. RNA sequencing was performed to examine the differentially expressed genes with Cu-TA treatment. Next, pathway analysis was performed to understand the functional significance of the altered gene expression. These results were compared to our published work on TA to see if Cu-TA is working in a similar mechanism. It also helped us to identify other underlying mechanisms. After identifying the major pathways affected, key regulators were confirmed by qPCR or Western blot assay using specific primers or antibodies.

MATERIALS AND METHODS

RNA Sequencing:

MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA using Cu-TA's IC50 value (29 μM) for 48 h. Cell lysates were collected and washed in PBS. Then 250 μl of HTG Molecular Lysis Buffer (HTG Molecular, Tucson, AZ) was added and mixed. Samples were heated to 95°C for 15 min and then stored at -80°C. Samples were later kept on dry ice and shipped to HTG Molecular Diagnostics at UT Southwestern Medical Center (Dallas, TX) for sequencing. RNA profiling of control (treated with vehicle) or Cu-TA treated cells was performed using HTG EdgeSeq Oncology Biomarker Panel. This Next-Generation Sequencing (NGS) assay uses quantitative nuclease protection (qNPA) to examine 2,560 markers that are tumor biology related. Using the HTG EdgeSeq processor, gene-specific DNA nuclease protection probes were added to samples to hybridize to target RNA. S1 nuclease was then added to remove all excess unhybridzed probes and RNA. This left a 1:1 ratio of probes and target RNA. Primers and tags were added and the remaining probes were then amplified by PCR. After removing excess primers, the products were pooled together, quantitated, and a sequencing library was created using an NGS platform. An overview of this NGS assay is depicted in Figure 13.

Ingenuity Pathway Analysis (IPA):

Pathway analysis was performed to determine the functional significance of the differentially expressed genes with Cu-TA treatment. Genes that had a fold change of > 1.5 over control were selected and entered into the IPA® software (Version: 43605602; Redwood City, CA) for expression analysis (Core Analysis module), and the program was used with its default settings. The IPA software contains an Ingenuity Knowledge Base. This is comprised of the most up-to-date knowledge on genes, pathways, regulators, etc. The IPA software uses its own algorithms to identify trends between the dataset and the Ingenuity Knowledge Base.

Western Blot:

MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA (29 μM). After 48 h, cells were harvested to prepare whole cell lysates. Total cellular protein was extracted using cell lysis buffer and protein quantification was done using the Pierce BCA Micro-Protein Assay Kit (Thermo Scientific, Waltham, MA). Protein samples were then separated through 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane. Next, the membranes were blocked with 5% milk in Tris-Buffered Saline with 1% Tween. Protein expression of TP53 (Cell Signaling Technology, Danvers, MA), ErbB2 (Thermo Scientific), Sp1 (Santa Cruz Biotechnology, Dallas, TX), and STAT3 (Cell Signaling Technology) were evaluated using specific antibodies while the expression of β-actin was used as a loading control. Blots were incubated with primary antibody overnight and incubated with secondary antibody for one hour the following day. Bands were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

Quantitative Polymerase Chain Reaction (qPCR):

MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA (29 μM) for 48 h. Cells were collected and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Total RNA was then synthesized to single-stranded cDNA using Superscript III (Invitrogen) and then amplified. Centromere protein F (CENPF), DNA damage inducible transcript 3 (DDIT3) and S-phase kinase-associated protein 2 (SKP2) were probed for using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA), GAPDH was used as a housekeeping gene. Samples were placed in a 96-well LightCycler 96 Real-Time PCR system (Roche) for qPCR analysis. Each sample was done in triplicates.

Statistical Analysis:

The statistical analyses to identify the genes that were differentially expressed following Cu-TA treatment in the RNA sequencing results were determined and provided by the HTG Molecular Diagnostics at UT Southwestern Medical Center (Dallas, TX). IPA was used with its built-in statistical module, and $p < 0.05$ considered significant.

RESULTS

The HTG EdgeSeq Oncology Biomarker panel consisting of 2,560 genes was used in this study. After analyzing the sequencing data of differentially expression of genes among control and Cu-TA treated samples, 436 genes were found to have >1.5 fold change over control and were selected for pathway analysis using the IPA software.

Networks affected by Cu-TA treatment: The Ingenuity Knowledge Base examines any relationships between genes in a given dataset. Sets of genes that are found to be connected are grouped into networks and the top 3 functions of this network is given. The differentially expressed genes in PaCa cells with Cu-TA treatment were found to be involved in a total of 18 networks (Table 2). These networks are: 1. Cell Morphology, Cellular Movement, Nervous System Development and Function; 2. Cancer, Hematological Disease, Immunological Disease; 3. Cell Cycle, Cell Death and Survival, DNA Replication, Recombination, and Repair; 4. Cell Death and Survival, Cancer, Organismal Injury and Abnormalities; 5. Cell Death and Survival, Cancer, Organismal Injury and Abnormalities; 6. Infectious Diseases, Cell Death and Survival, Cancer; 7. Cell Cycle, Cell Death and Survival, Cancer; 8. Immunological Disease, Cancer, Organismal Injury and Abnormalities; 9. Cell Cycle, Cancer, Organismal Injury and Abnormalities; 10. Dermatological Diseases and Conditions, Organismal Injury and Abnormalities, Digestive System Development and Function; 11. Endocrine System Disorders,

Gastrointestinal Disease, Metabolic Disease; 12. Cancer, Hematological Disease, Organismal Injury and Abnormalities; 13. Cardiovascular System Development and Function, Cell Cycle, Cell Morphology; 14. Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function; 15. Cancer, Hematological Disease, Immunological Disease; 16. Cell Death and Survival, Inflammatory Disease, Inflammatory Response; 17. Cellular Movement, Cellular Development, Cellular Growth and Proliferation; 18. Cardiac Arteriopathy, Cardiovascular Disease, Organismal Injury and Abnormalities. These networks were associated with cancer and related diseases broadly and, also, implicating specific causative links (e.g., cell death and survival, immunological disease, organismal injury and abnormalities, cellular growth and proliferation), feature (morphology), and biological processes (system development and function, cell cycle, DNA replication, recombination and repair, cellular movement, inflammatory response, etc.). All 18 individual network figures are shown in the appendix.

Overlapping networks: Overlapping networks contain genes that are found in both networks. Only 2 networks were found to be independent, thus, Figure 14 shows 16 of the 18 networks involved with Cu-TA treatments. It also gives the three overlapping and interconnected networks. The #1 overlapping networks is made up of 12 networks (2, 3, 4, 6, 7, 9, 10, 11, 13, 14, 15, 16), the #2 overlapping networks contains 2 networks (1, 18), and the #3 overlapping networks also has 2 networks (8, 17). Figure 15 shows a visual representation of the #1 overlapping networks merged into one picture to illustrate complexity. The dense lines indicate the overlapping connections and similarities between various networks. Upregulated and downregulated genes were colored in green and red, respectively.

Regulators involved with Cu-TA-induced gene alterations: IPA analysis revealed the up-stream regulators that are partially responsible for the differentially expressed genes observed. The top 10 up-stream regulators include: TP53 (tumor protein p53), TNF (tumor necrosis factor), ErbB2 (human epidermal receptor growth factor 2), TGFB1 (transforming growth factor beta-1), Sp1 (specificity protein 1), TP63 (tumor protein 63), RELA (transcription factor p65), NFkB (nuclear factor kappa B), AR (androgen receptor) and STAT3 (signal transducer and activator of transcription 3) (Table 3). Their p-value is also given along with their predicted activation status. The top 10 networks and network functions that were affected were also presented in Table 3. All of the networks were given a score based on how many molecules in that network were affected. Cell cycle, cell death, and cancer were recurring terms among the most prevalent network functions listed.

In order to confirm the sequencing results, the change in the protein expression of important regulators was determined by Western blot analysis for TP53, ErbB2, Sp1, and STAT3 (Figure 16). The protein expression (Western blot results) correlated with pathway analysis results. The protein expression of TP53 was found to be increased with Cu-TA treatment while there was a downregulation in ErbB2, Sp1, and STAT3.

A closer look at a selected network: Since, we observed induction of apoptosis and inhibition of anti-apoptotic markers with Cu-TA treatment, we selected network #7 (Figure 17**)** which has several such markers for further observation to see the changes in the molecules involved in the network. Among the molecules affected are survivin (BIRC5), PARP, and caspase. The up-regulated markers were colored in green and the down-regulated markers in red. The changes observed in these molecules further confirm the activation apoptosis.

Top associated molecular and cellular functions affected: Several molecular and cellular functions in the PaCa cells were influenced with the treatment. The top 4 functions are: cell death and survival, cellular development, cell growth and proliferation, and cellular movement. Table 4 shows this list alongside their corresponding *p*-values. The number of molecules affected (#Molecules) is also shown and is out of the 436 genes put into the IPA software.

Confirmation of selected genes via qPCR: The HTG sequencing results gave a number of differentially expressed genes. Among them were CENPF, DDIT3 and SKP2; all of which were included in the list of genes submitted for expression analysis by IPA. CENPF is a component of the centromere-kinetochore complex and aids in chromosomal segregation during cell cycle progression. Overexpression of CENPF has been found to contribute to pathogenesis in a number of cancers [47]. DDIT3 is a transcription factor involved with activation of apoptosis during stress caused to the endoplasmic reticulum [48]. SKP2 is one of the proteins that comprise the Skp1–Cullin1–F-box (SCF) E3 ligase complex involved with targeting molecules for degradation to transition into S-phase in the cell cycle [49]. A high expression of SKP2 is associated with metastasis and poor prognosis in PaCa patients [50]. These three genes were selected because of their involvement in apoptosis and cell cycle in cancer progression. Additionally, 'cell cycle' and 'cell death' were reoccurring networks in the list of Top Networks (Table 2). CENPF, DDIT3 and SKP2 were probed for qPCR analysis in MIA PaCa-2 cells treated with Cu-TA and these results were then compared to our previous pathway analysis study using TA's IC_{50} value [27] (Figure 18A&B). Both TA and Cu-TA treatments had a similar trend in results: CENPF and SKP2 were found to be significantly downregulated and DDIT3 was found to be upregulated. However, Cu-TA had a considerably greater increase in DDIT3 than TA, about 20-fold greater.

DISCUSSION

PaCa is a lethal malignancy with a low 5-year survival rate. Patients are typically diagnosed once the cancer is advanced and metastatic, making them ineligible for surgery [6, 51]. Since this malignancy is usually at advanced state at the time of diagnosis, chemotherapy and radiation are not effective. Thus, PaCa urgently requires more successful and sensitizing agents for treatment. Our laboratory and others have shown the potential of the small molecule TA as an anti-cancer agent for a variety of different malignancies, including PaCa. Recently, we demonstrated that when TA is prepared as Cu-TA, it results in enhanced activity against PaCa cell growth when compared to TA. We also showed that Cu-TA was stable both in physical state (structure) and anti-cancer activity for up to 1 year and not toxic to non-malignant (cardiomyocytes) cells. In this aim, we used NGS to assess differentially expressed genes with Cu-TA in PaCa cells and then used IPA analysis to understand their functional significance.

Pathway analysis revealed 18 networks associated with Cu-TA treatment in PaCa cells. There were a large number of overlapping networks and molecular/cellular functions, showing that these networks are interdependent. The more networks that are interconnected suggests that the genes and genes' functions are biologically significant. Thus, since cell cycle, cell death and cancer were reoccurring network functions, this indicates that they are important biological processes to Cu-TA treatment. The top up-stream regulators revealed molecules responsible for the genetic alterations involved with Cu-TA treatment. Among these regulators were TP53, ErbB2, Sp1, and STAT3. TP53 encodes the tumor suppressor protein p53, which is involved with various processes including induction of cell cycle and apoptosis and DNA repair [52]. Mutated TP53 is one of the most common mutations found in human cancers, including pancreatic [53]. The MIA PaCa-2 cell line used in this study also has a mutation in TP53, affecting its activity [54]. Studies testing anti-cancer agents in MIA PaCa-2 cells have seen an

upregulation in TP53 expression with treatment and established this increase in expression also increased its activity [55, 56]. One study in particular demonstrated that p53 was modified at a residue located in its sequence-specific DNA binding domain after treatment, and this increased the protein's stability and activity [57]. Activation of the previously mutated p53 protein could potentially be occurring with Cu-TA treatment as well, thus, restoring its tumor suppressor function. ErbB2 is part of the epidermal growth factor receptor family and acts as a co-receptor with other receptor family members [58]. Upon dimerization and activation, ErbB2 regulates processes such as proliferation and migration [59]. ErbB2 overexpression has been found in PaCa and this expression results in a poorer prognosis [60, 61]. As previously mentioned, Sp1 is a transcription factor found to be overexpressed and contribute to the progression of PaCa [26, 28]. STAT3 is a transcription factor responsible for regulating several genes involving cell proliferation, survival, apoptosis, and invasion [62]. STAT3 has been found to be constitutively active in several malignancies, including PaCa [63, 64]. Hyperactivation of STAT3 contributes to tumor progression and resistance to treatment [65, 66]. Thus, these four regulators were selected for confirmation because of their involvement in PaCa. ErbB2, Sp1, and STAT3 were all found to be downregulated with Cu-TA treatment. Because these proteins are found to be overexpressed in PaCa and contribute to proliferation, their downregulation may be partially responsible for the anti-proliferative effect seen with Cu-TA. Moreover, the selected Network #7 (Figure 17) is also significant as survivin (BIRC5), PARP, and effector caspases are affected by Sp1 and STAT3. Survivin transcription is regulated both by Sp1 and STAT3 [30, 67]. PARP is a DNA repair protein that becomes cleaved and, thus, inactivated by effector caspases during apoptosis [68]. Thus, the downregulation seen with Sp1 and STAT3 can perhaps induce apoptosis by decreasing survivin (inhibitor of apoptosis protein) expression to allow caspase cleavage to occur, also resulting in cleaved PARP. Western blot results corroborate the pathway

analysis and HTG sequencing findings and demonstrate that these regulators are the ones largely involved with Cu-TA treatment in PaCa cells.

Additionally, the top 4 molecular and cellular functions affected included cell death and survival, cellular development, cell growth and proliferation, and cellular movement. This further demonstrates Cu-TA's potential as an anti-cancer agent, as it targets the biological functions needed for an anti-tumor effect. This also correlates with the top regulators confirming their involvement with cell growth, migration, apoptosis, survival, and cell cycle. The genes selected for confirmation by qPCR were chosen because of their functional significance, their involvement in cell cycle and apoptosis, and to compare to previous results with TA. In an earlier study, we performed molecular profiling delineating the pathway analysis identifying the altered gene expression and associated pathways induced by TA in MIA PaCa-2 cells [27]. While, 50 μM dose was used for TA treatment, only 29 μM dose was used for Cu-TA suggesting that Cu-TA is effective even at low doses. The confirmation qPCR results done in this study had a similar trend to our previous study for TA (upregulation for CENPF and SKP2, downregulation for DDIT3). This suggests that TA and Cu-TA are perhaps working in a similar mechanism affecting cell cycle and apoptosis genes. However, there was an enhanced effect seen with SKP2 and especially DDIT3 upon Cu-TA treatment. Cu-TA had a 20-fold change in upregulating DDIT3 compared to TA. Therefore, qPCR results also demonstrate that Cu-TA has an enhanced effect and at lower doses than TA. DDIT3 upregulation, having the most predominant increase compared to CENPF and SKP2, suggests that Cu-TA is most likely affecting apoptotic pathways in MIA PaCa-2 cells.

These next-generation sequencing (NGS) and pathway analysis results provides an insight on the myriad of different networks, pathways, and biological and molecular functions that are

affected in Cu-TA treated PaCa cells. Cell death and survival, cellular growth and proliferation, and cellular movement are all affected functions in PaCa cells, which are important for cancer cells to progress and metastasize. As presented in the schematic (Figure 19), the overall analyses of all results (sequencing, qPCR and Western blot) demonstrate the effect of Cu-TA on molecular markers involved in critical cellular and biological processes that are associated with cell survival (downregulation; red color) or apoptosis (upregulation; green color). These results along with our previous studies further confirm Cu-TA's potential as an anti-tumor agent for PaCa. Furthermore, these results are also beneficial for a better comprehension on pancreatic tumor biology and perhaps novel potential therapeutic targets for PaCa.

FIGURES

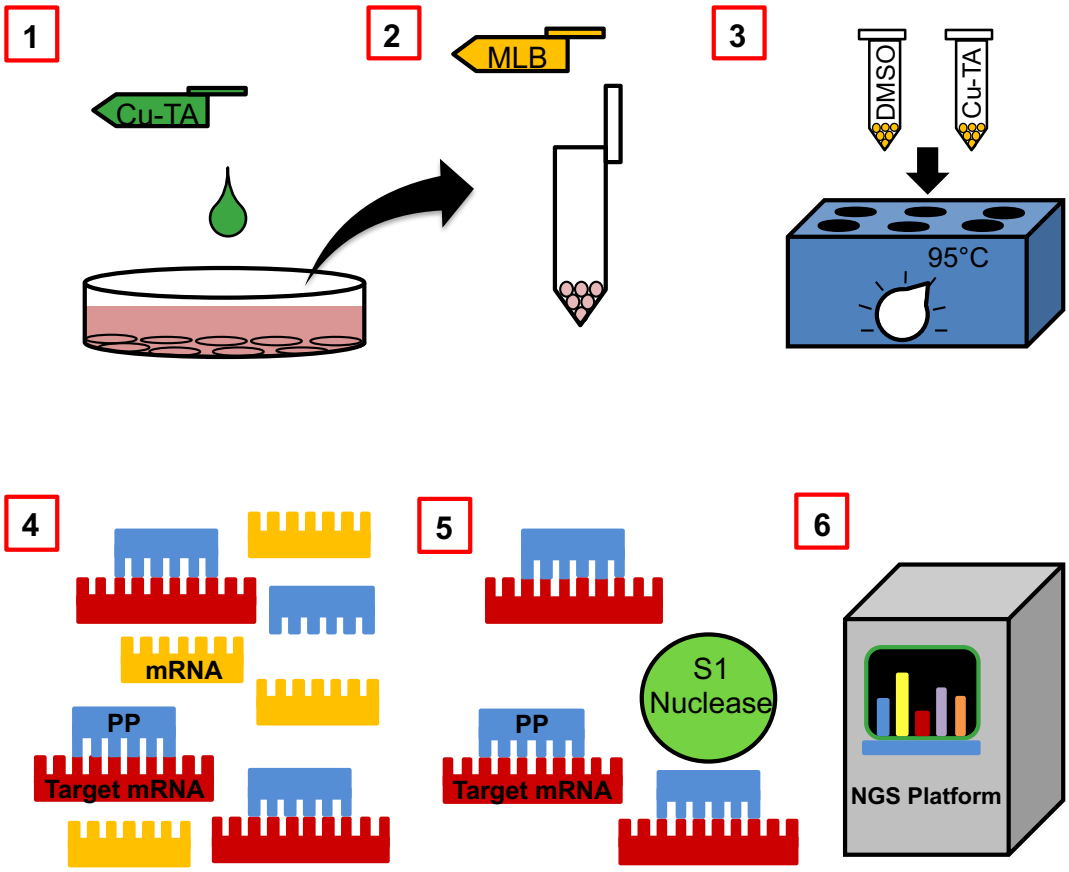


Figure 13. Overview of RNA Sequencing Assay. 1) PaCa cells were treated with either DMSO (control) or Cu-TA for 48 h. 2) Cells were then collected and HTG Molecular Lysis Buffer (MLB) was added and mixed. 3) Samples were heated for 15 min at 95°C. Samples were then kept on dry ice and shipped to HTG Molecular Diagnostics. The HTG EdgeSeq Oncology Biomarker Panel was selected to look at Cu-TA's effect on tumor biology markers. 4) Protection probes (PP) were added to samples and allowed to hybridize to their target mRNA. 5) S1 nuclease was added to remove excess PPs and unhybridized mRNA, leaving PPs with their target RNA in a 1:1 ratio. 6) Primers and tags were then added to amplify the remaining probes by PCR. Lastly, the products were quantitated and an NGS platform was used to make a sequencing library.

#	Top Networks	Score
1	Cell Morphology, Cellular Movement, Nervous System Development and Function	35
2	Cancer, Hematological Disease, Immunological Disease	35
3	Cell Cycle, Cell Death and Survival, DNA Replication, Recombination, and Repair	33
4	Cell Death and Survival, Cancer, Organismal Injury and Abnormalities	31
5	Cell Death and Survival, Cancer, Organismal Injury and Abnormalities	30
6	Infectious Diseases, Cell Death and Survival, Cancer	30
7	Cell Cycle, Cell Death and Survival, Cancer	30
8	Immunological Disease, Cancer, Organismal Injury and Abnormalities	28
9	Cell Cycle, Cancer, Organismal Injury and Abnormalities	26
10	Dermatological Diseases and Conditions, Organismal Injury and Abnormalities, Digestive System Development and Function	26
11	Endocrine System Disorders, Gastrointestinal Disease, Metabolic Disease	26
12	Cancer, Hematological Disease, Organismal Injury and Abnormalities	26
13	Cardiovascular System Development and Function, Cell Cycle, Cell Morphology	24
14	Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function	18
15	Cancer, Hematological Disease, Immunological Disease	15
16	Cell Death and Survival, Inflammatory Disease, Inflammatory Response	15
17	Cellular Movement, Cellular Development, Cellular Growth and Proliferation	13
18	Cardiac Arteriopathy, Cardiovascular Disease, Organismal Injury and Abnormalities	9

Table 2. Top Networks: ID Associated Network Functions. Gene expression correlation with the top networks affected after 48 h of Cu-TA treatment in PaCa cells. The score indicates how many molecules are affected.

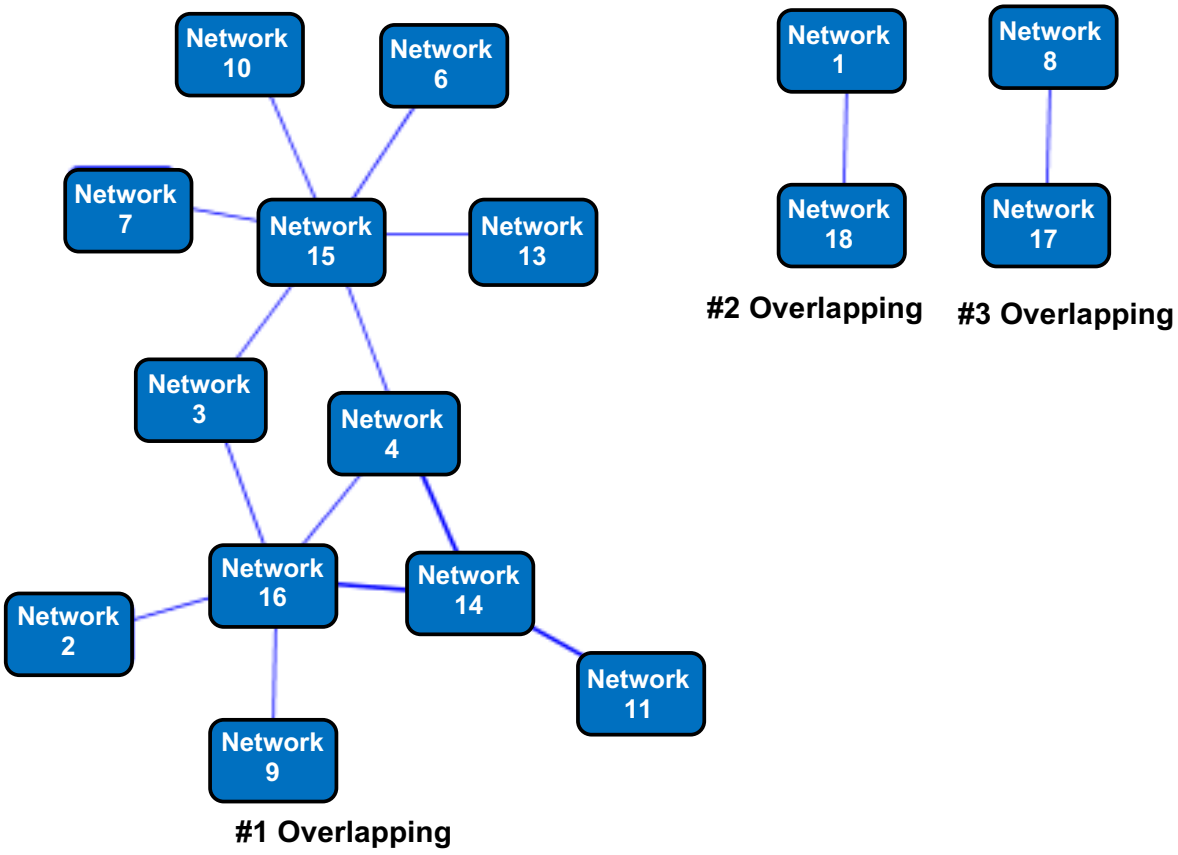


Figure 14. Overlapping networks. Out of the 18 networks affected, 16 are displayed in this figure. There are 3 groups of networks and within each group they have overlapping genes and thus, functions.

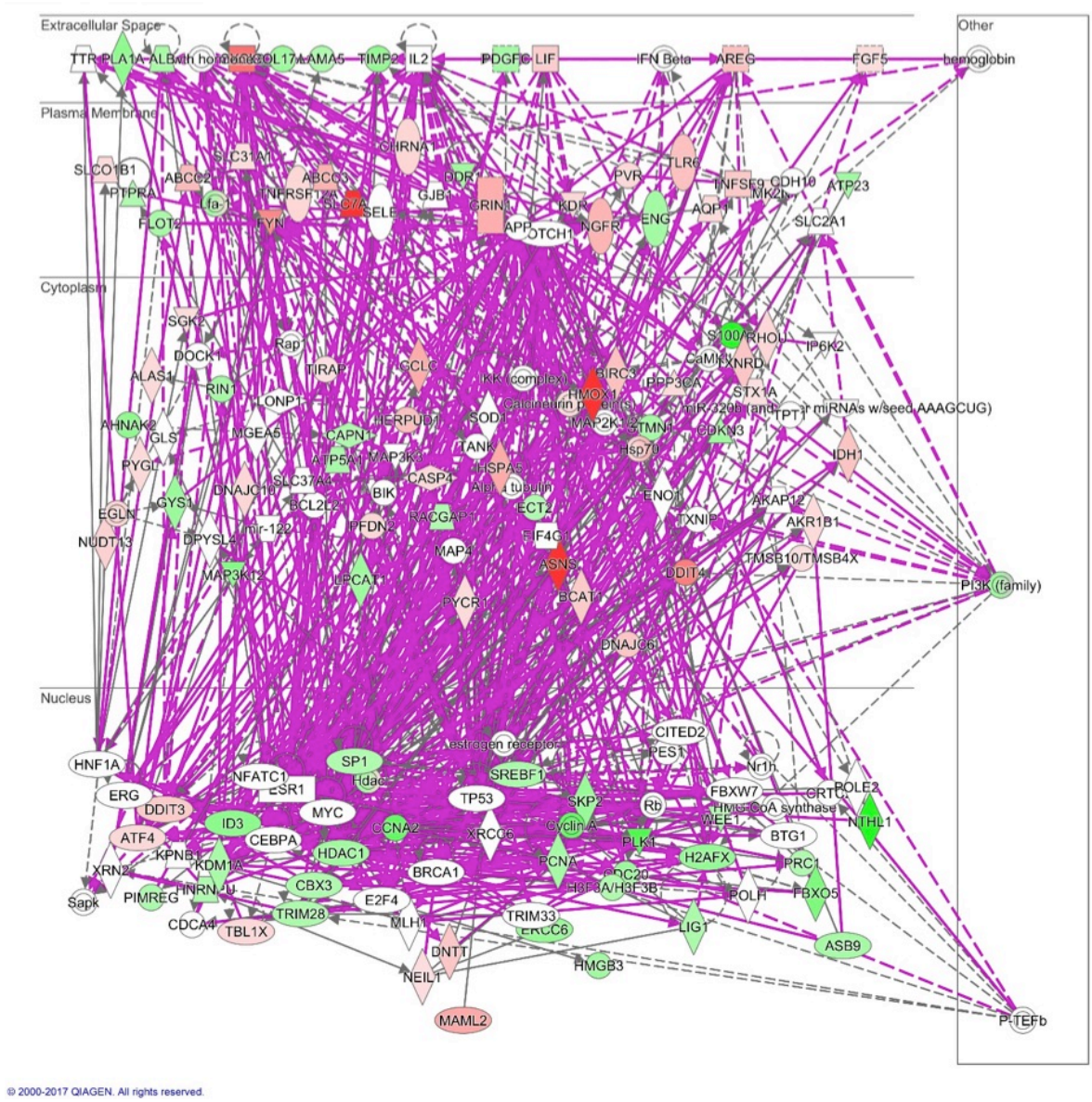


Figure 15. **Merged Networks (Core of #1 Overlapping Networks).** 16 networks were found to have overlapping genes. This shows the core of #1 overlapping networks, 12 networks (2, 3, 4, 6, 7, 9, 10, 11, 13, 14, 15, 16).

#	Upstream Regulators	*p*-value of overlap
1	TP53	1.26E-39
2	TNF	1.98E-35
3	ERBB2	8.76E-30
4	TGFB1	5.86E-28
5	SP1	5.64E-25
6	TP63	3.9E-23
7	RELA	9.06E-23
8	NFkB (complex)	2.45E-21
9	AR	1.12E-20
10	STAT3	5.89E-20

Table 3. Top Upstream Regulators. A list of the top upstream regulators involved with the genetic alterations induced by Cu-TA 48 h post-treatment. They are all found to be statistically significant and the *p*-value is < 0.001.

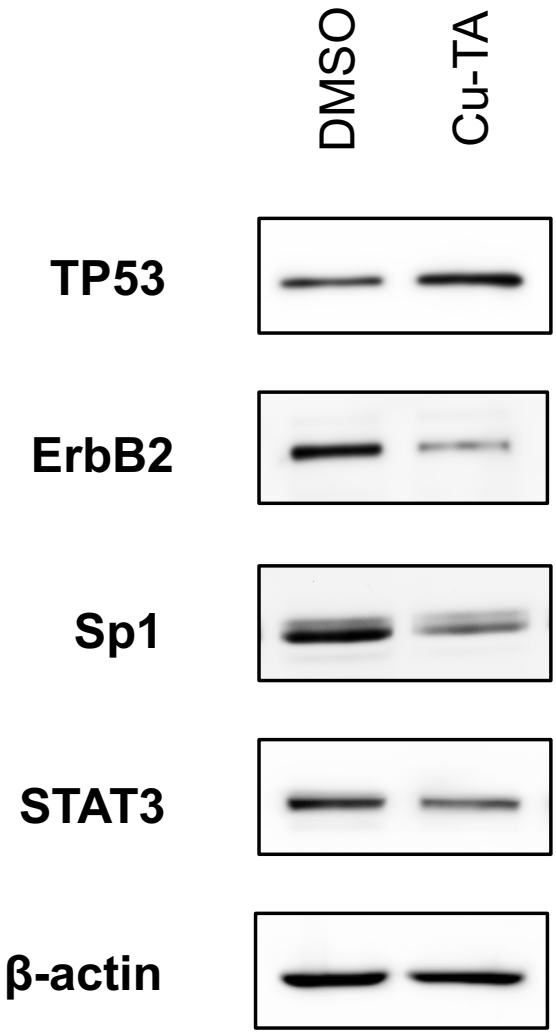


Figure 16. Top Regulators affected by Cu-TA treatment. MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA (29 μM) for 48 h. Cell lysates were prepared and then protein expression of TP53, ErbB2, Sp1, and STAT3 was then determined by Western blot analysis. β-actin as used as a loading control.

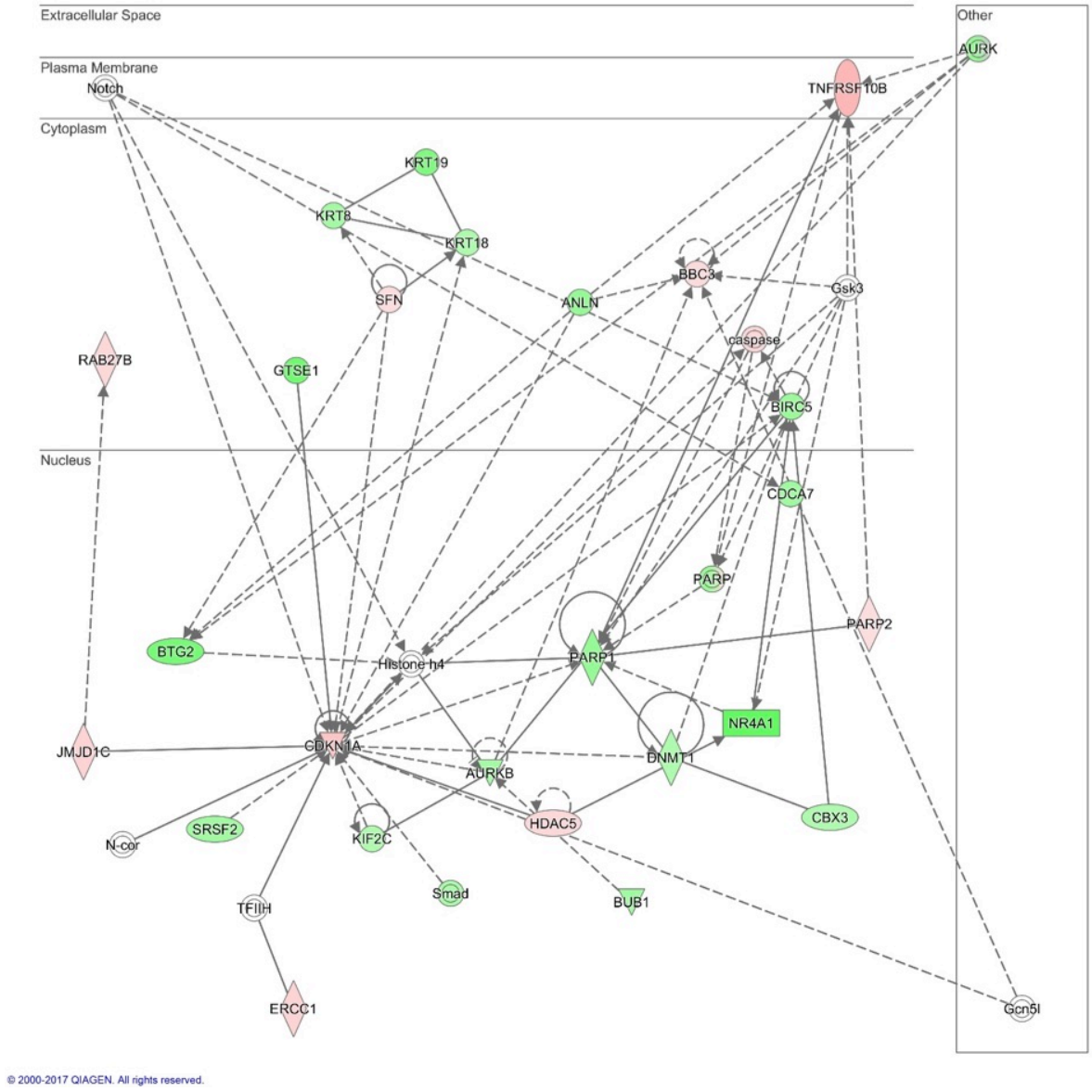
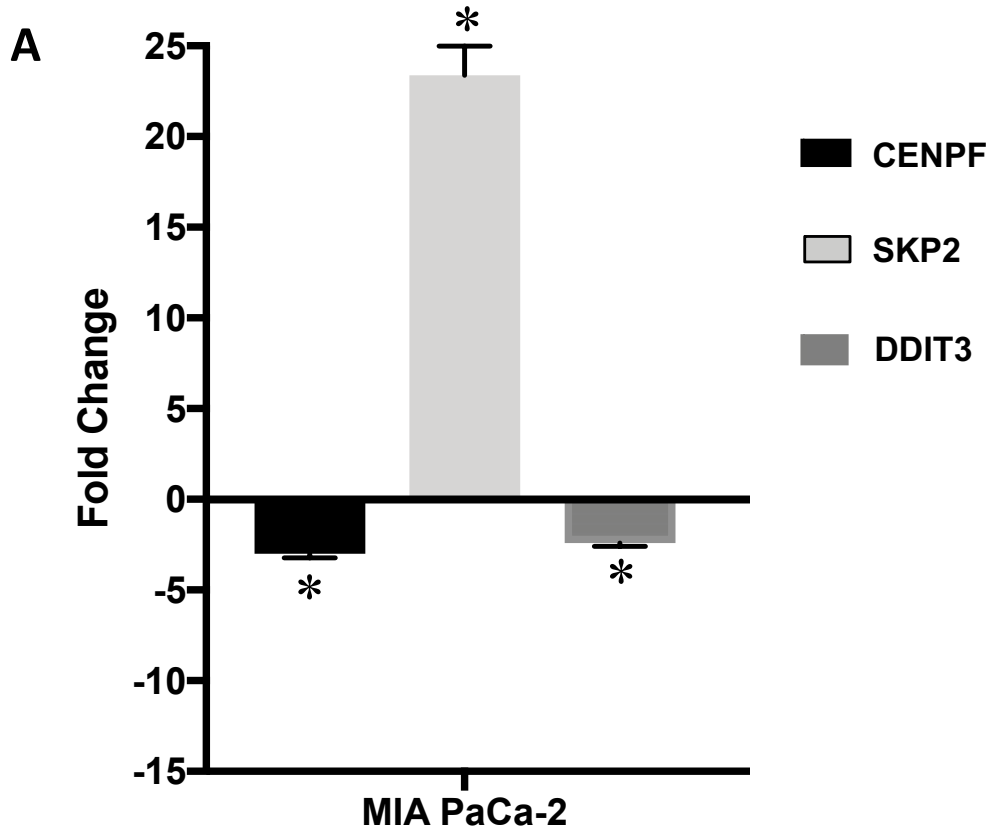


Figure 17. Selected network for further analysis #7. Network #7 was selected to show the molecules involved in this particular network. The molecules in green represents a downregulation while red signifies an up-regulation. The intensity of the color correlates to the amount of down or up-regulation.

Name	*p*-value	#Molecules
Cell Death and Survival	1.69E-11 – 6.03E-82	261
Cellular Development	3.72E-11 – 3.67E-64	220
Cellular Growth and Proliferation	3.72E-11 – 3.67E-11	232
Cellular Movement	2.20E-11 – 1.77E-44	165

Table 4. Top diseases and bio functions: Molecular and cellular functions. The top 4 biological functions altered by Cu-TA treatment are given. #Molecules indicates how many molecules in that biological function are affected. The *p*-value is < 0.001.



B

Comparison of TA and Cu-TA		
Gene	TA (50 μM)	Cu-TA (29 μM)
CENPF	-4.1	-3.0
DDIT3	2.4	23.39
SKP2	-2.1	-2.41

Figure 18. mRNA level fold change of selected genes by TA or Cu-TA treatment. (A) MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA (29 μM) for 48 h. mRNA expression levels of CENPF, DDIT3 and SKP2 were then probed for while GAPDH was used as a loading control. Data shown gives the fold change of genes normalized to the control. All treatments were done in triplicate, and the bars marked with '*' are significantly different from corresponding control ($p < 0.05$). (B) This data was then compared to our results from a previous pathway analysis study using TA's IC_{50} value.

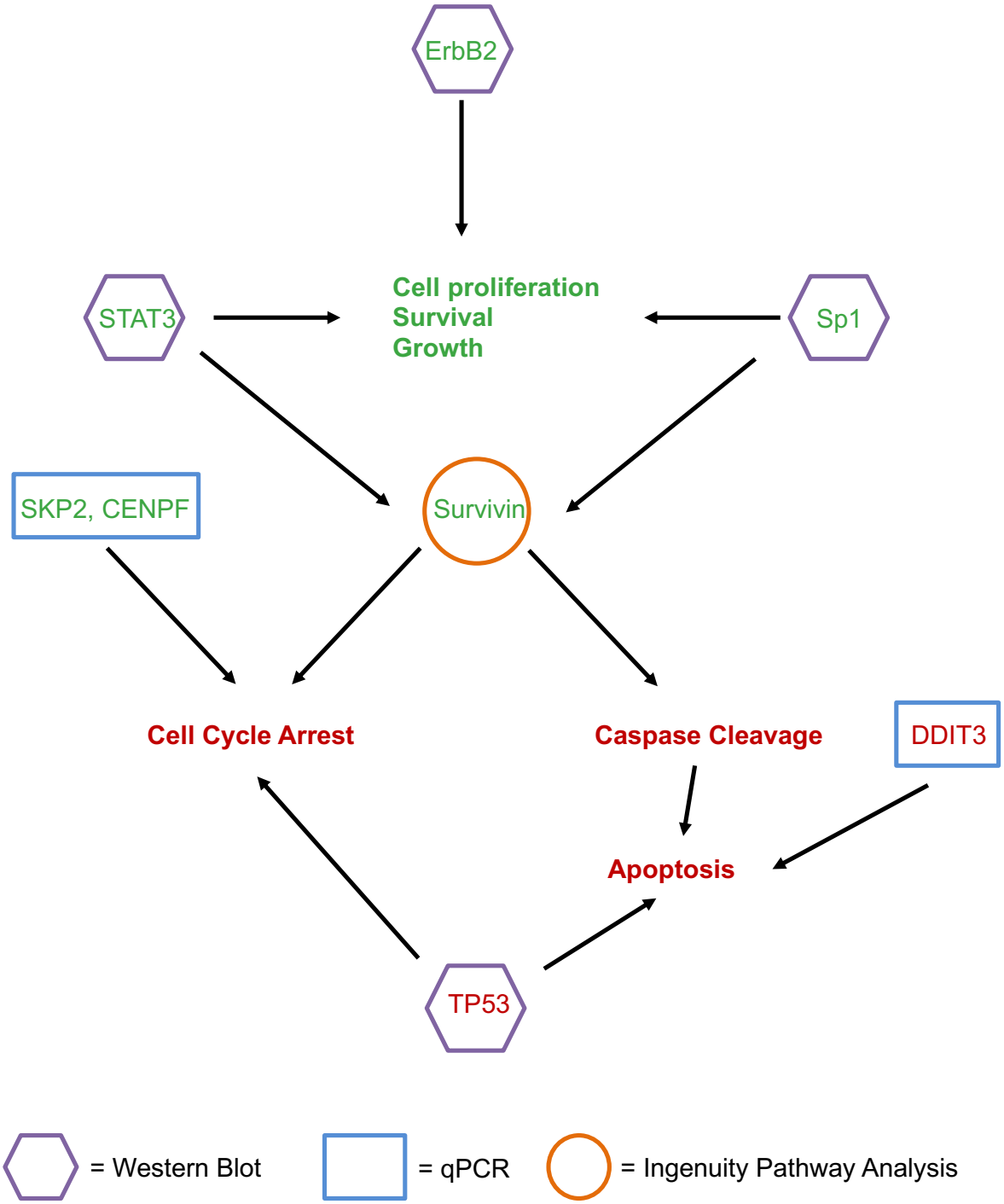


Figure 19. Schematic diagram illustrating Cu-TA's biological targets in MIA PaCa-2 cells. The schematic diagram showing Cu-TA's mechanism of action is based on the Ingenuity Pathway Analysis, Western blot and qPCR results. Targets are outlined in a shape given in the legend while cellular and biological processes are given in bold. Text in red indicates upregulation while green indicates downregulation.

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CHAPTER – VI

SUMMARY AND FUTURE PERSPECTIVES

SUMMARY

Despite medical advancements, PaCa unfortunately still remains a lethal malignancy. Patients are typically diagnosed once the cancer is advanced and metastatic, making them ineligible for surgery [1, 2]. The cancer is usually aggressive at the time of diagnosis, so chemo and radiation offer little benefit. Therefore, PaCa urgently requires more effective and sensitizing agents for treatment. Two targets of interest for PaCa have been transcription factors Sp1 and Sp3. Both Sp1 and Sp3 are involved with regulating cell proliferation, differentiation and apoptosis [3]. Their overexpression has been found to contribute to the progression, advancement, and poor prognosis of many types of cancers, including pancreatic [4-6]. Survivin, an inhibitor of apoptosis protein is known to be regulated by both of these Sp proteins [7]. Survivin is highly expressed during fetal development, but in adult cells its expression is marginal [8]. Survivin has also been found to be overexpressed in various tumor types and adds to the cancer's resistance to cytotoxic therapies [9, 10]. For these reasons, Sp1, Sp3, and survivin have been targets of interest for PaCa and researchers have becoming interested in finding drugs that inhibit their expression. Consequently, the small molecule TA has been gaining popularity for its anti-cancer properties such as inhibition of cell growth and induction of apoptosis in various tumor models [11-14]. TA has been shown to work by downregulation of Sp proteins and survivin [15, 16]. TA has also been demonstrated to sensitize PaCa cells to radiation treatment [17].

Although the results seen with TA thus far seem promising, its IC_{50} value is slightly high. Thus, we began investigating a derivative of TA (Cu-TA) in an effort to enhance its activity in PaCa cells (MIA PaCa-2 and Panc1). In this dissertation, Cu-TA was found to have an anti-proliferative effect in PaCa cells and its IC_{50} value was half that of TA's. Characterization and activity testing of Cu-TA demonstrated its stability (up to 12 months) and intact biological (anti-proliferative) activity. Importantly, treatment of Cu-TA on cardiomyocytes did not cause cytotoxicity. This is significant since NSAIDs can potentially cause cardiotoxicity. Cu-TA also downregulates expression of Sp proteins and survivin and the molecular markers involved with PaCa growth and progression. Additionally, Cu-TA induces apoptosis and cell cycle arrest in PaCa. Finally, RNA sequencing and subsequent pathway analysis of treatment revealed Cu-TA affects pathways involved with cancer progression and metastasis.

After the completion of the specific aims in this study and conducting a through literature review, it seems that multiple additional mechanisms could be associated with the anti-tumoral response seen with Cu-TA (Figure 19). Cu possesses pro-oxidant activity and induces reactive oxygen species, which leads to cytotoxicity [18]. Cu (II) ions are known to cause cytotoxicity in cancer cells by interfering with iron-sulfur clusters in proteins and conformational changes in metallo-proteins by displacing metals such as Zinc [19-21]. Copper(II) complexes with the ligands such as bis(thiosemicarbazone) were tested in several cancer models [22-24], while CuII(gtsm) [glyoxalbis(N4methylthiosemicarbazonato)CuII] was very effective at reducing cancer burden and lesion grade in an animal model (TRAMP) for prostate cancer [18]. Zhang et al. demonstrated that Cu (II) complexes (3-indolecarboxylic acid) target the 20S proteasome at the β5 subunit and cause the deactivation of the proteasome in cancer cells as well as anti-cancer activity [25]. Cu(II)-containing species are believed to act as anti-inflammatory agents [23, 26-28] by modulating the synthesis of prostaglandins [29-31]. TA is known to inhibit Sp1,

Sp3 and survivin, and these properties may be either retained or even improved when complexed with Cu (Cu-TA). These proteins participate in multiple vital functions including the activities that can impact apoptosis and/or cell cycle arrest. Cu(II)-containing products can also exhibit immunomodulatory responses that affect the expression of cytokines (e.g., interleukin IL-2) [32]. Thus, the addition of Cu enhances the cytotoxic activity of TA against cancer cells.

FUTURE DIRECTIONS

The ultimate goal of investigating Cu-TA would be to use this alongside standard treatment care to perhaps sensitize the cancer cells. Therefore, allowing the patients to receive less chemo and radiation for less side effects without compromising therapeutic activity. Future studies would include combination experiments; Cu-TA with chemotherapy or radiation. If Cu-TA is a successful sensitizing agent, then it would have a synergistic or additive effect with standard treatment. The Cu-TA's pathway analysis results could potentially be used to help identify the best suited chemotherapy agent to use in the combination experiments. The next steps to strengthen these studies would then be to repeat these experiments using patient-derived (PDx) cells and xenograft model. Many studies seem to be successful in preclinical studies, but once they reach translational testing they are often ineffective [33]. Using a PDx model can help address this issue as it provides a more reliable tumor model [34, 35]. Previously, we have shown that TA is effective in inducing apoptosis in PaCa cells as well as diminishing tumor size and growth in a mouse model [36]. We also measured the mice body weights, examined vital organs, performed a histopathology and found that there was no apparent toxicity [36]. Conducting similar studies with this new compound will enable us to further evaluate Cu-TA's effectiveness as an anti-cancer agent and any potential toxicity. The long-term goal of these studies would be to shift into clinical testing.

FIGURE

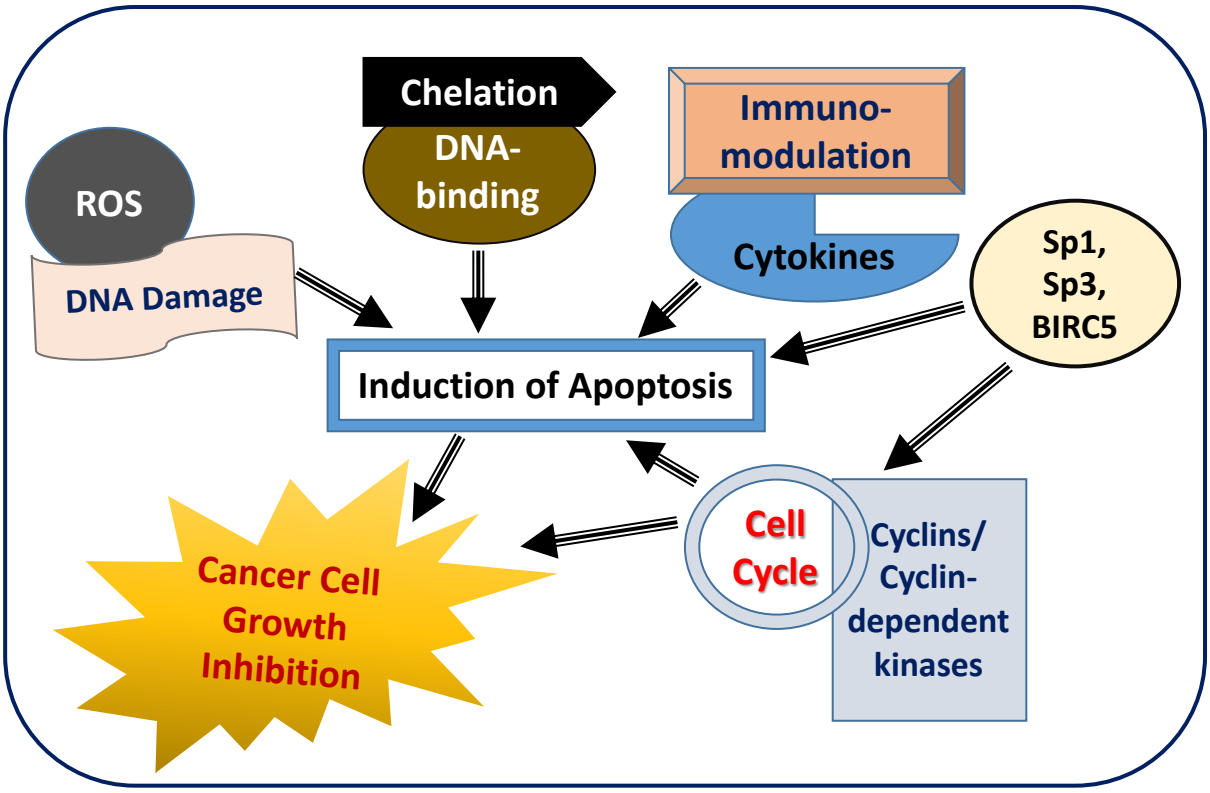

Figure 20. Additional potential mechanisms that may cause the anti-cancer activity of Cu-TA: Exploring the published work on Cu(II)-containing products in cancer, it is postulated that multiple mechanisms could be associated with the anti-cancer activity of Cu-TA. For example, induction of Reactive Oxygen Species (ROS), chelating effect disrupting the DNA-binding, immunomodulation (eg., cytokines) can induce apoptosis. Inhibition of Sp1, Sp3 and survivin can cause both induction of apoptosis and cell cycle arrest, while the direct/in-direct effect on cyclins and/or cyclin-dependent kinases can lead to cell cycle arrest ultimately leading to cell death or growth inhibition.

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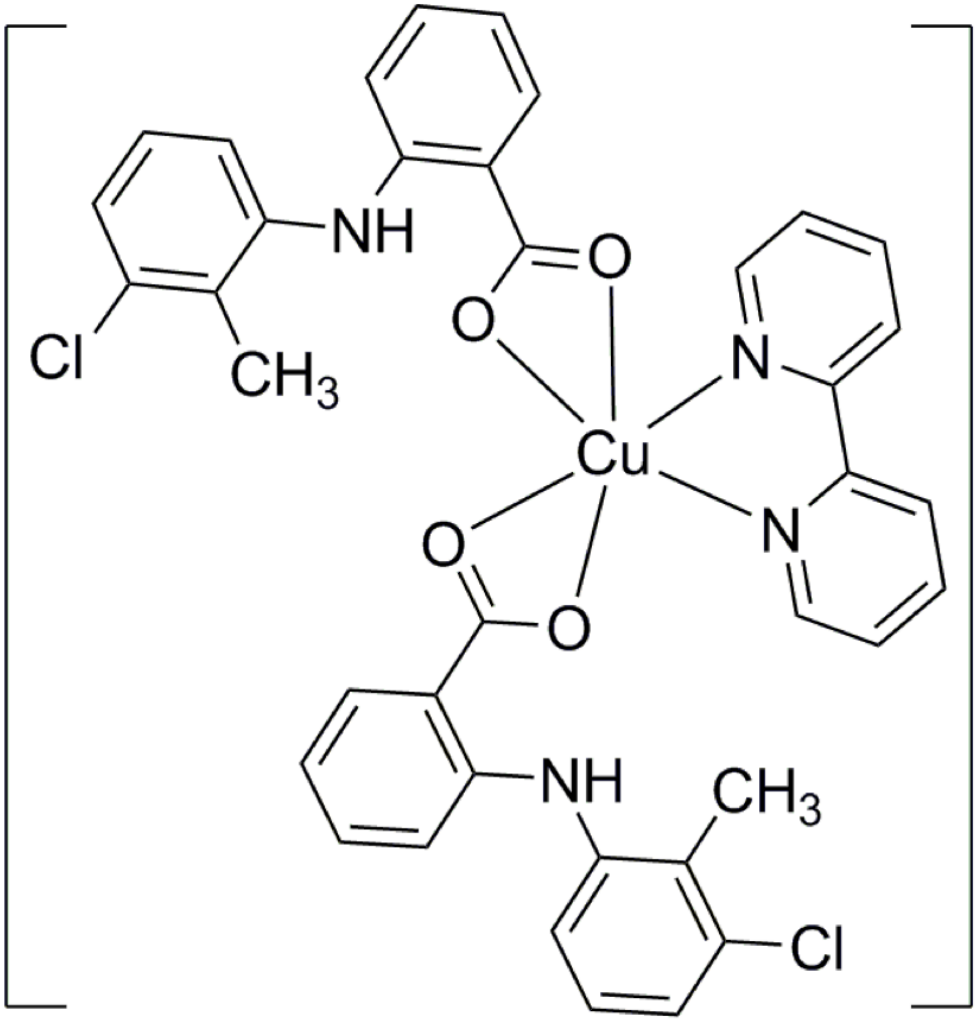
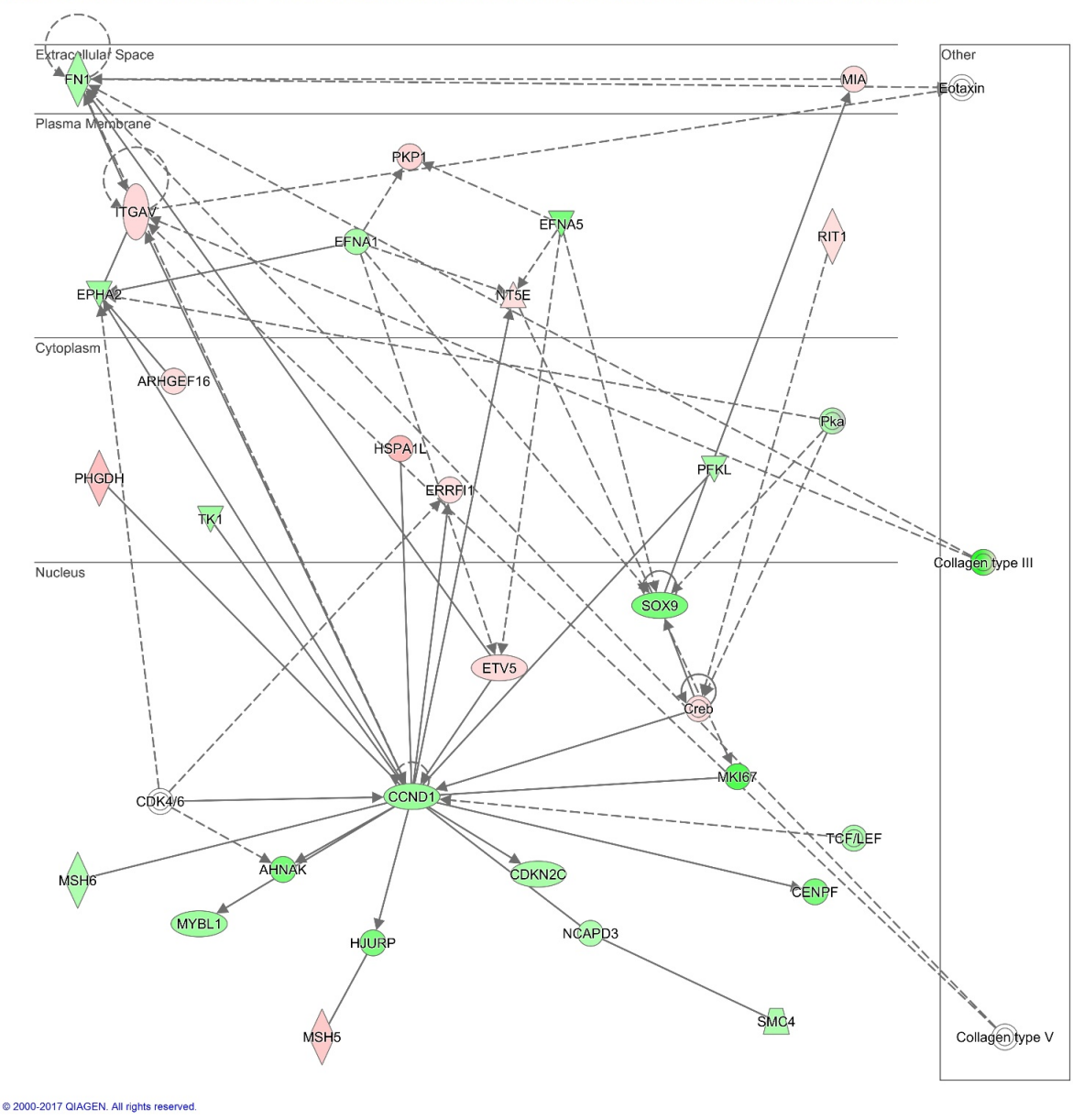
APPENDIX

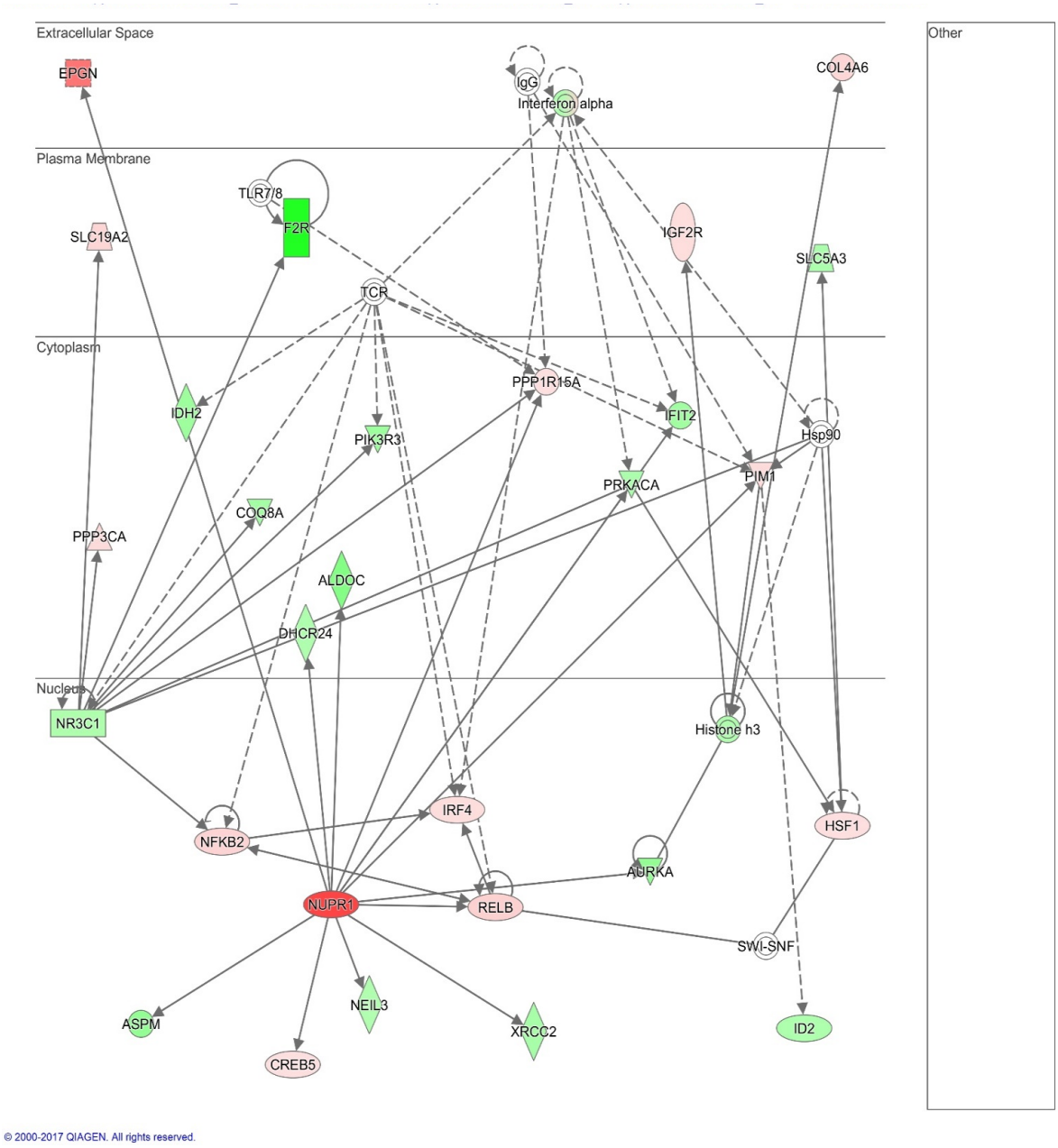
STRUCTURE OF CU-TA



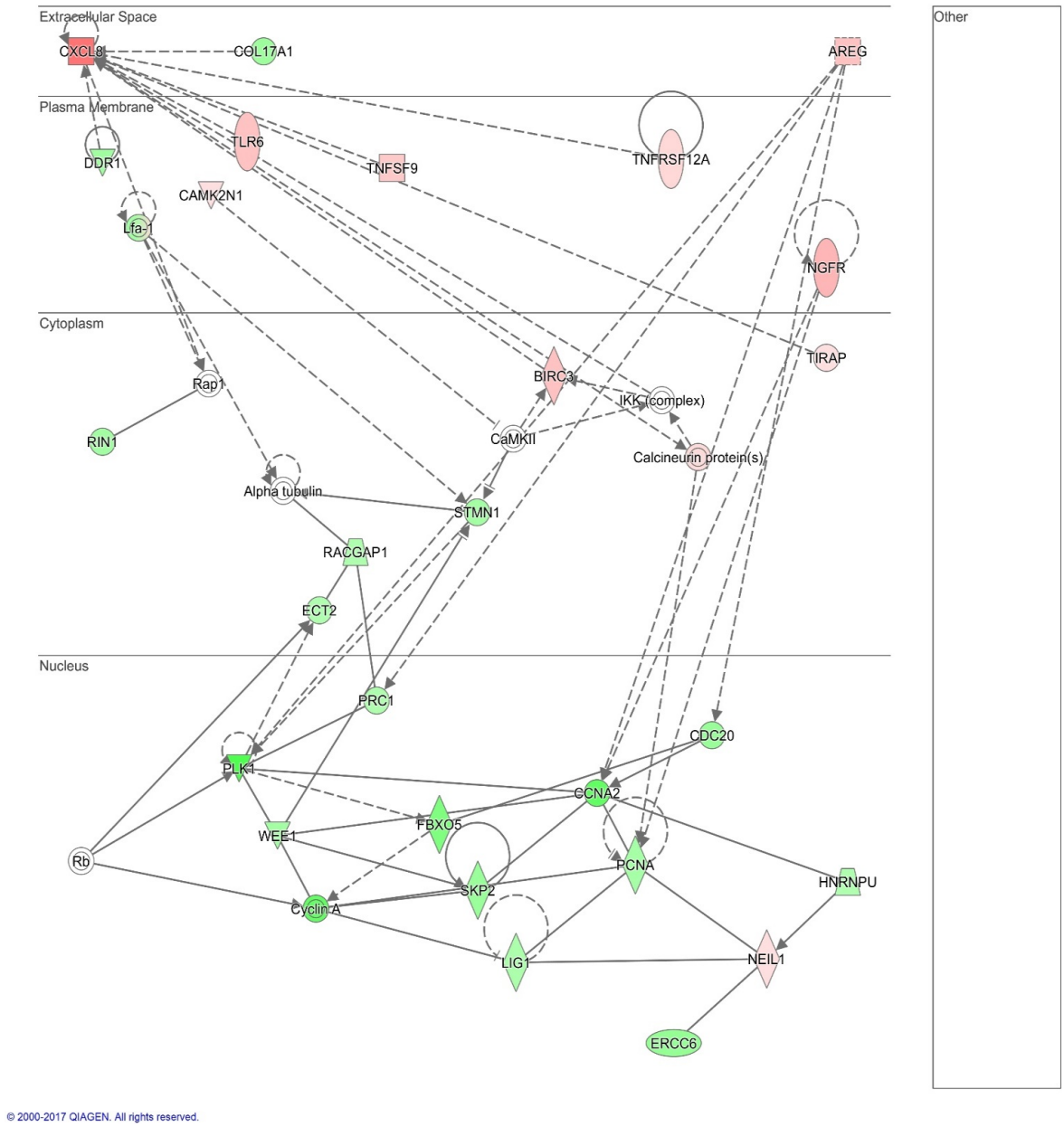
ALL 18 NETWORKS ASSOCIATED WITH CU-TA'S ANTI-CANCER ACTIVITY



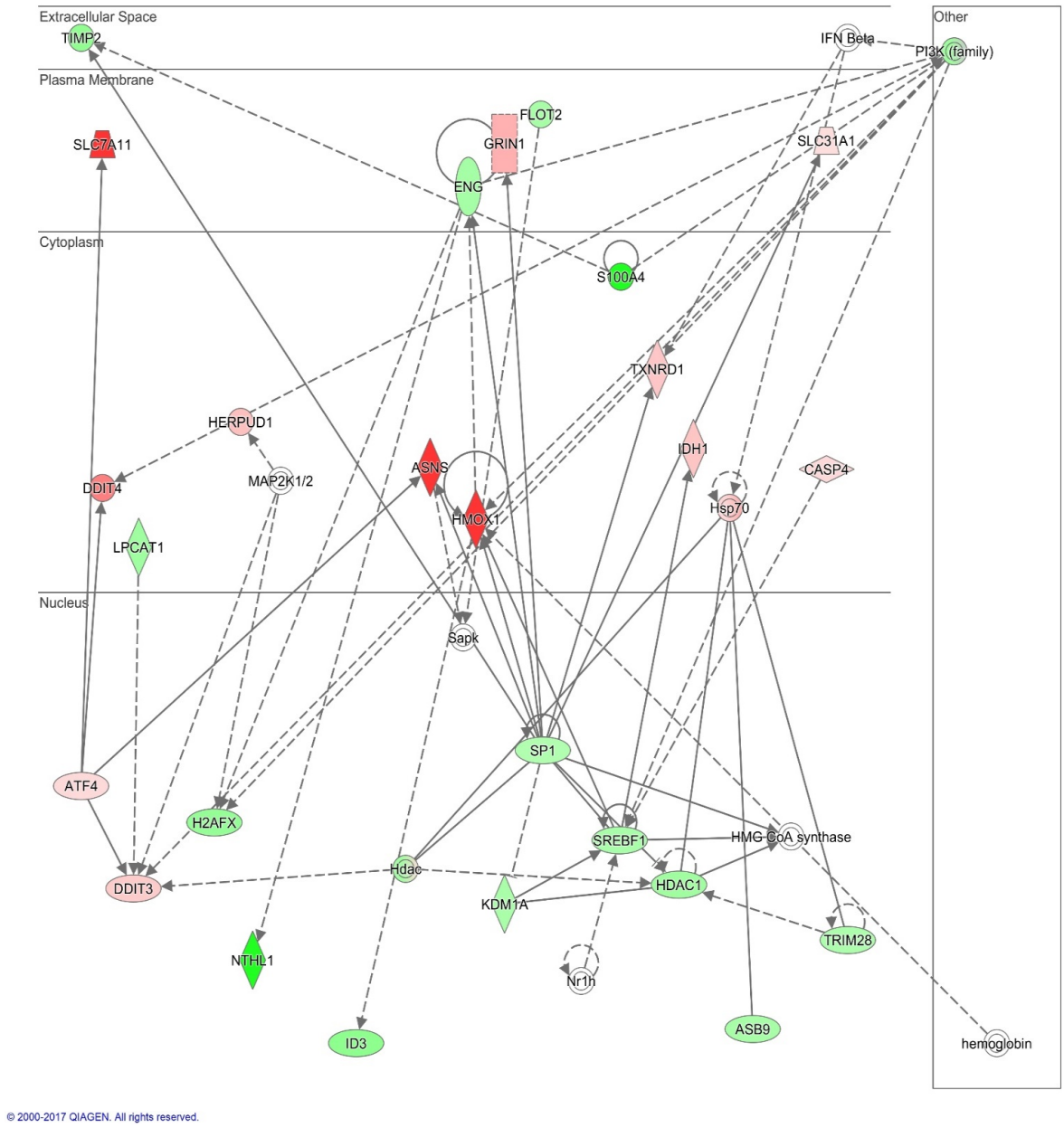
Network 1. Cell Morphology, Cellular Movement, Nervous System Development and Function.



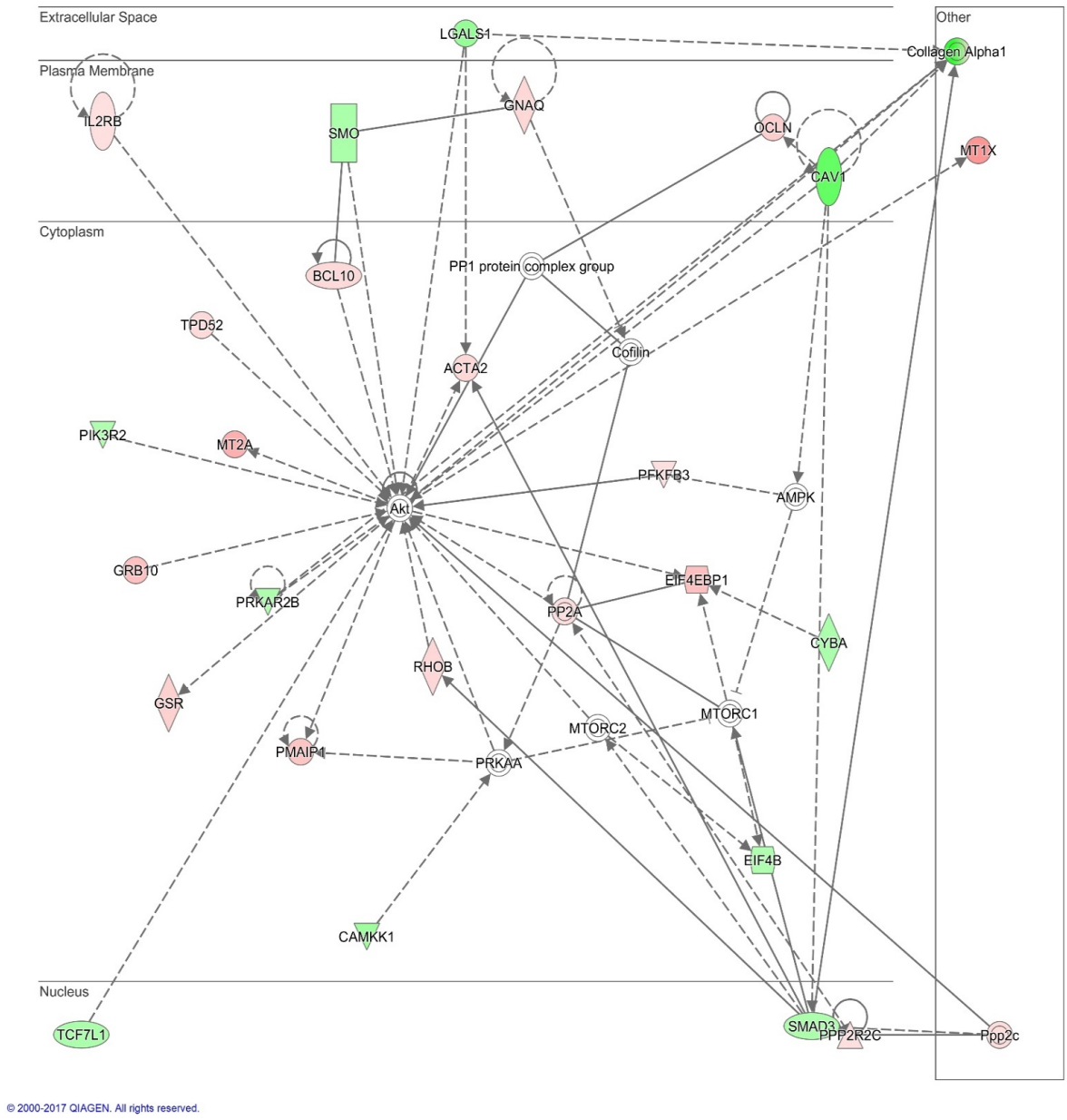
Network 2. Cancer, Hematological Disease, Immunological Disease.



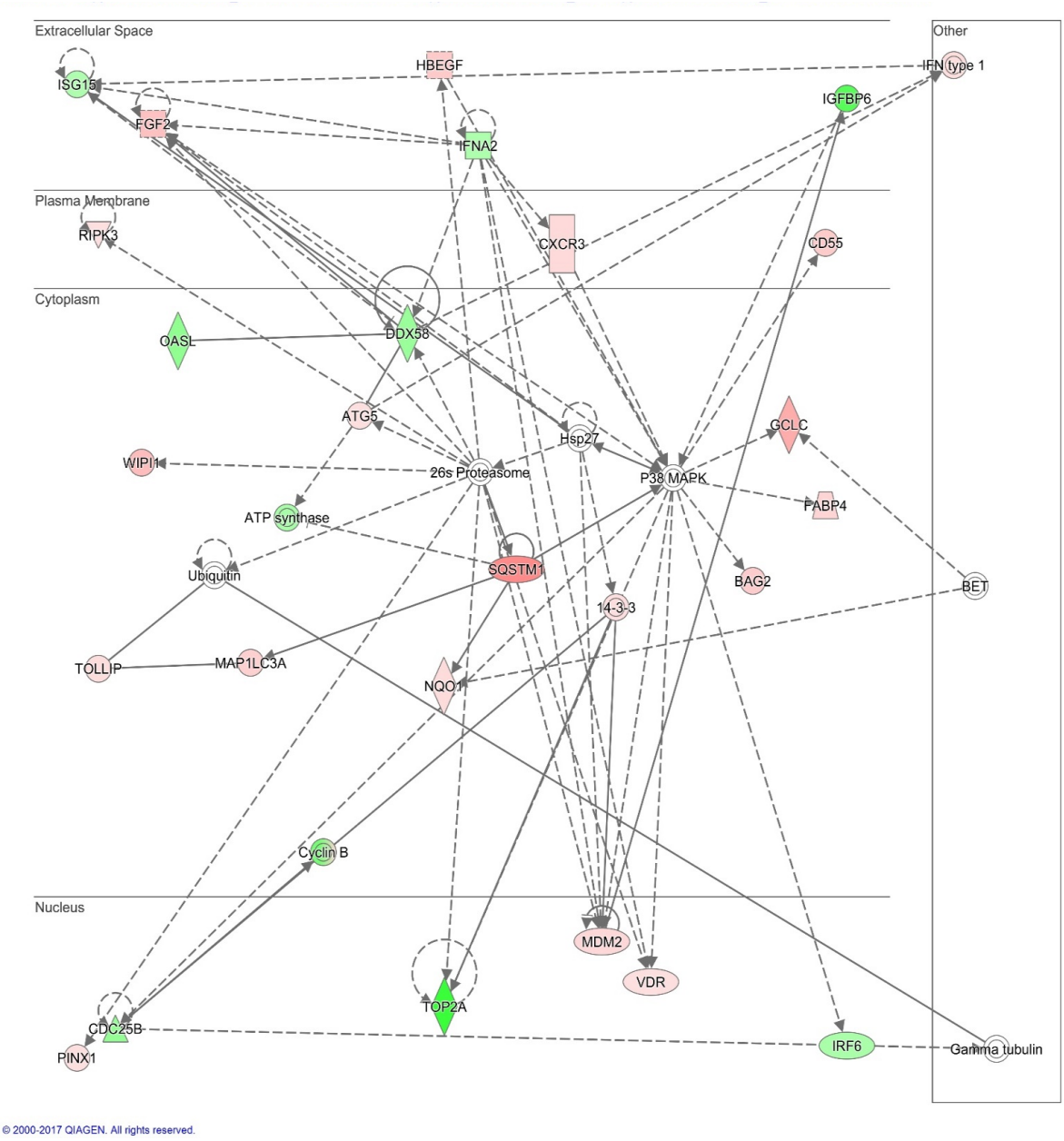
Network 3. Cell Cycle, Cell Death and Survival, DNA Replication, Recombination, and Repair.



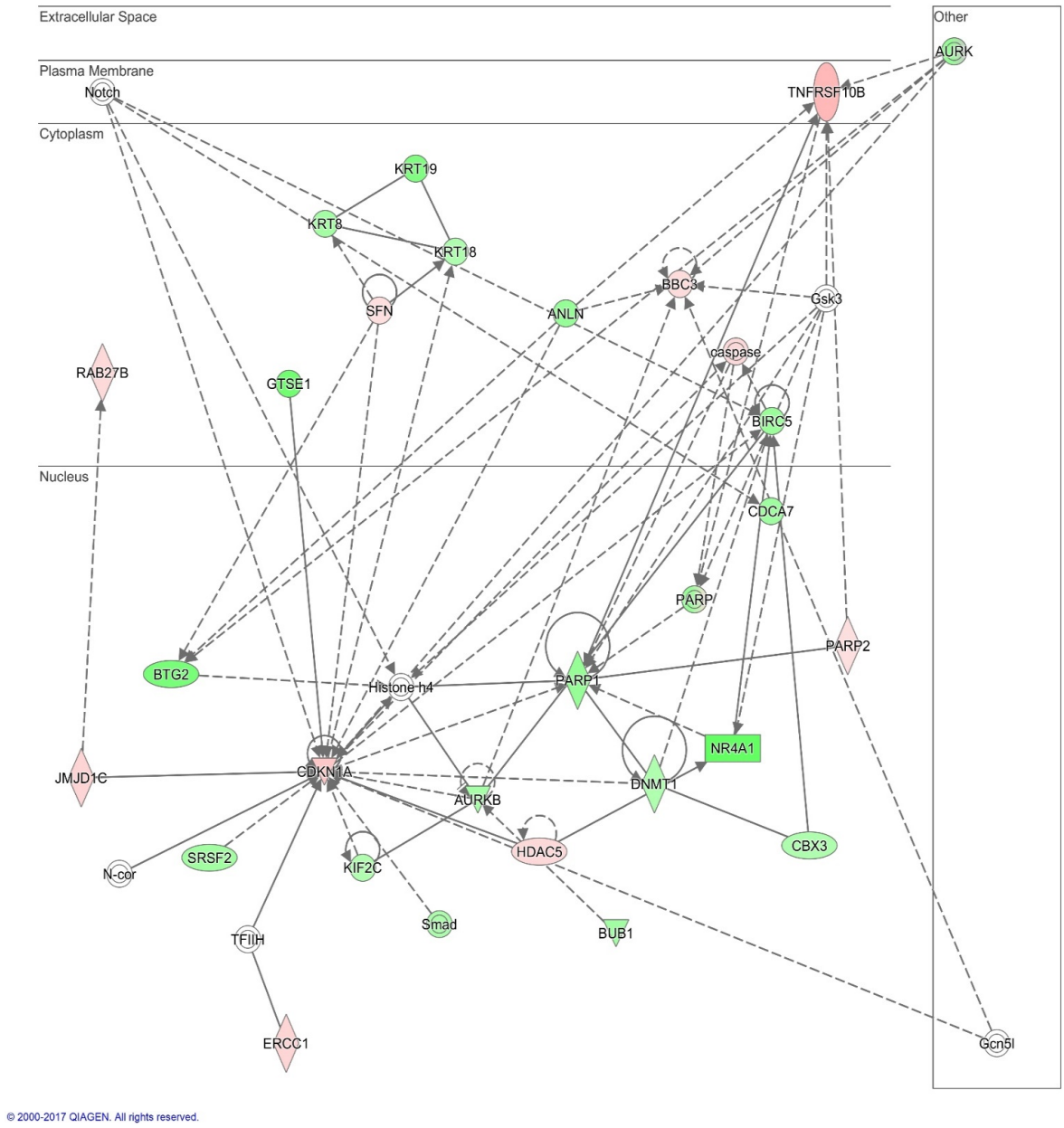
Network 4. Cell Death and Survival, Cancer, Organismal Injury and Abnormalities.



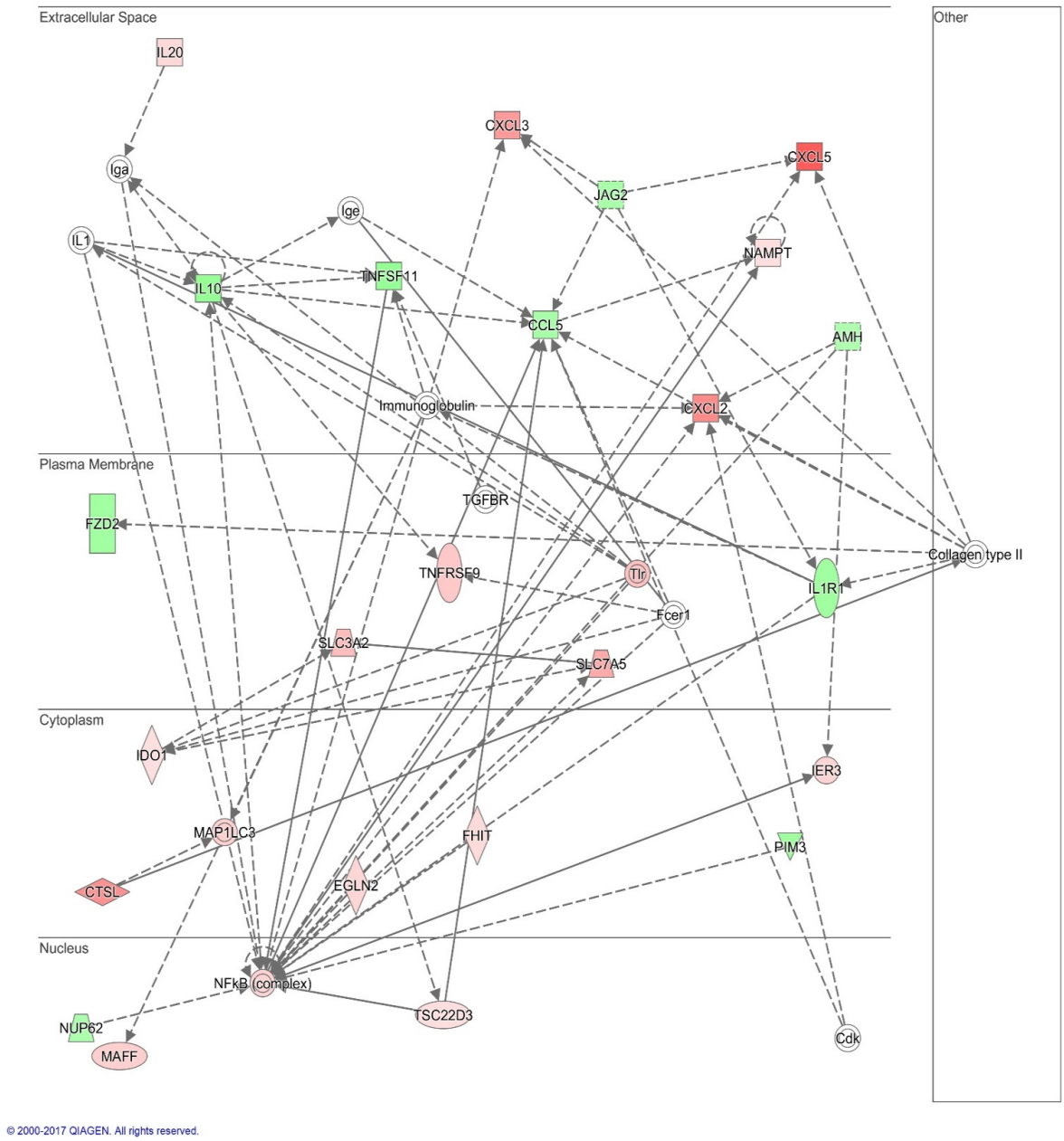
Network 5. Cell Death and Survival, Cancer, Organismal Injury and Abnormalities.



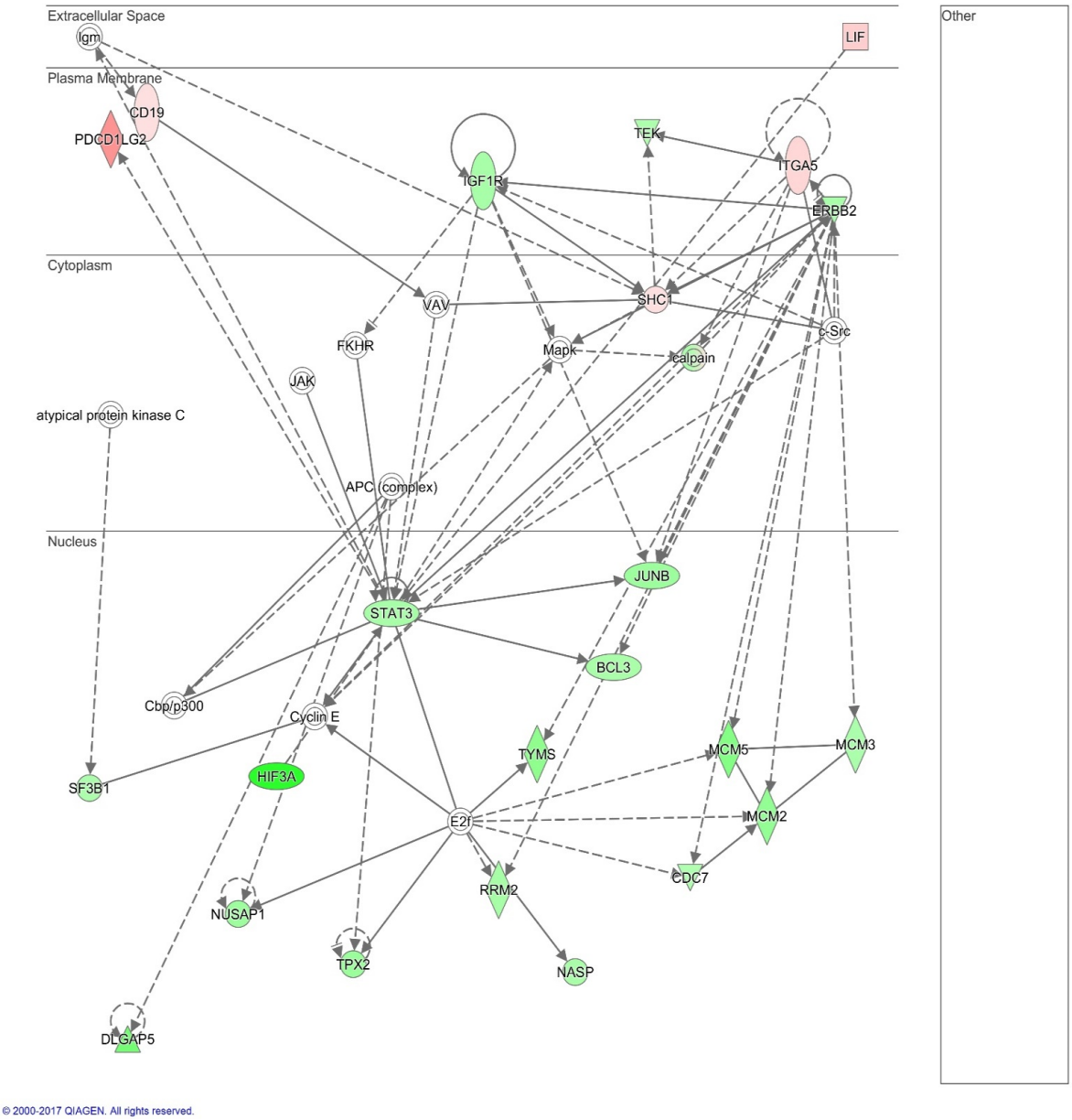
Network 6. Infectious Diseases, Cell Death and Survival, Cancer.



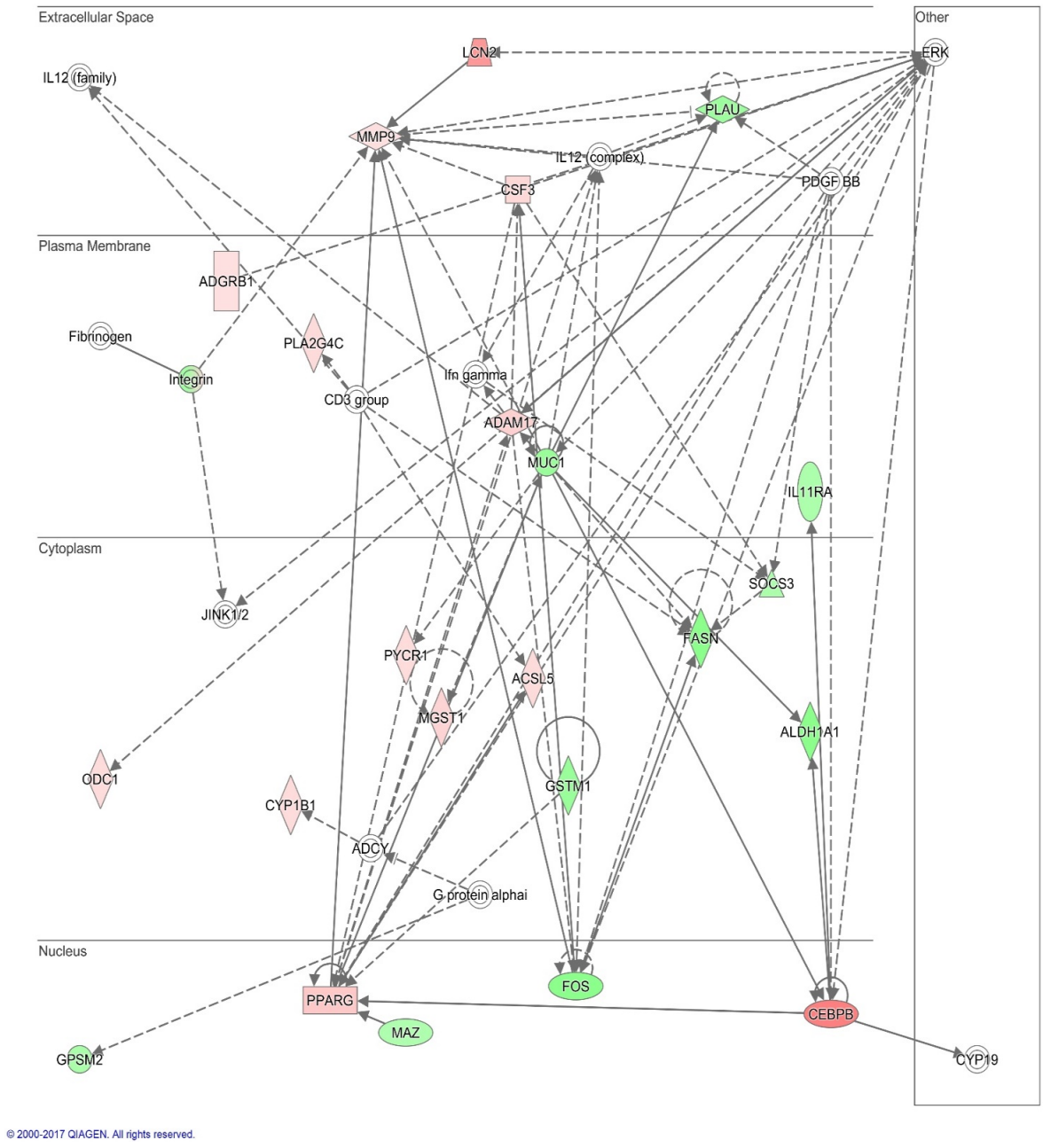
Network 7. Cell Cycle, Cell Death and Survival, Cancer.



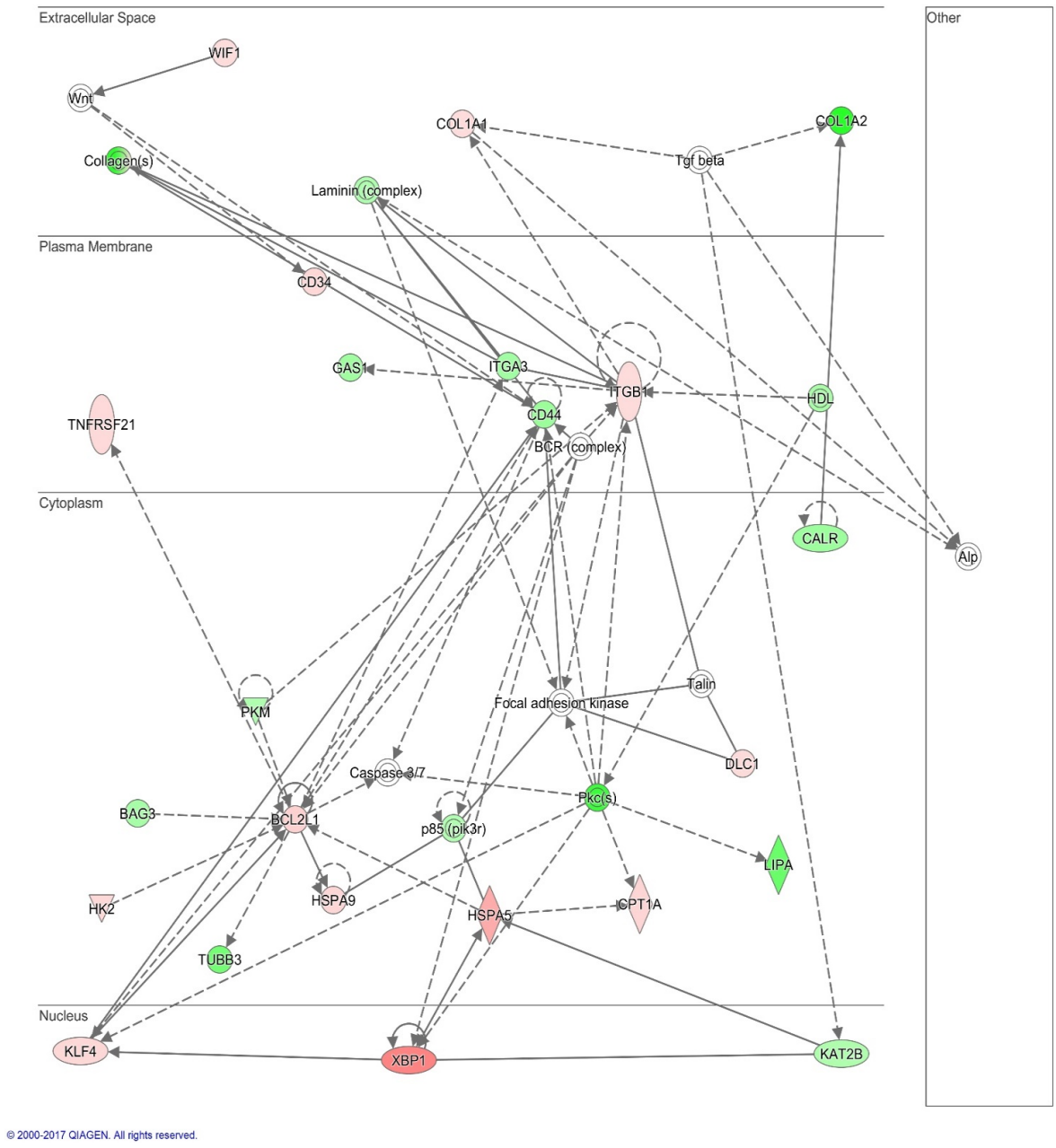
Network 8. Immunological Disease, Cancer, Organismal Injury and Abnormalities.



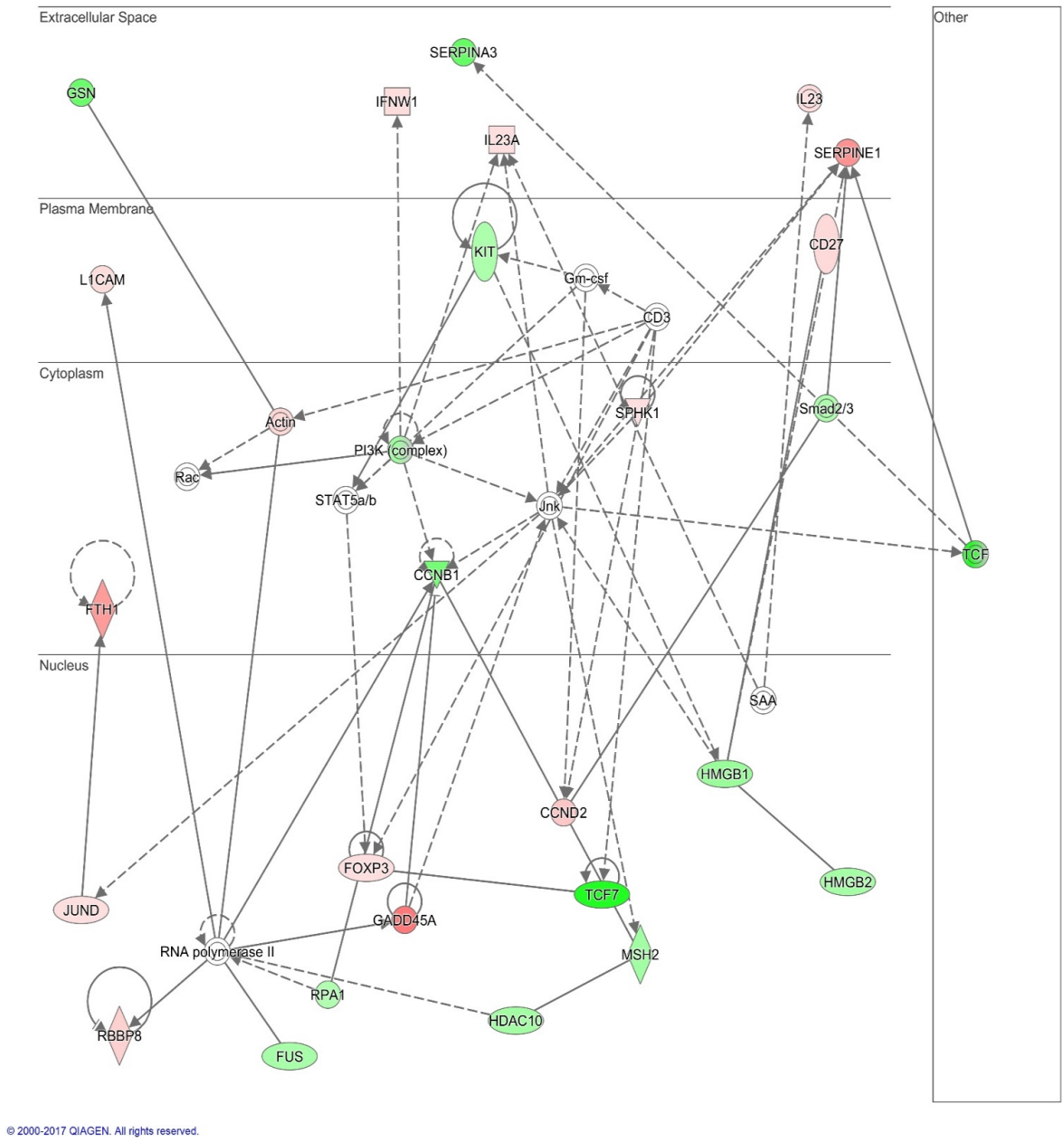
Network 9. Cell Cycle, Cancer, Organismal Injury and Abnormalities.



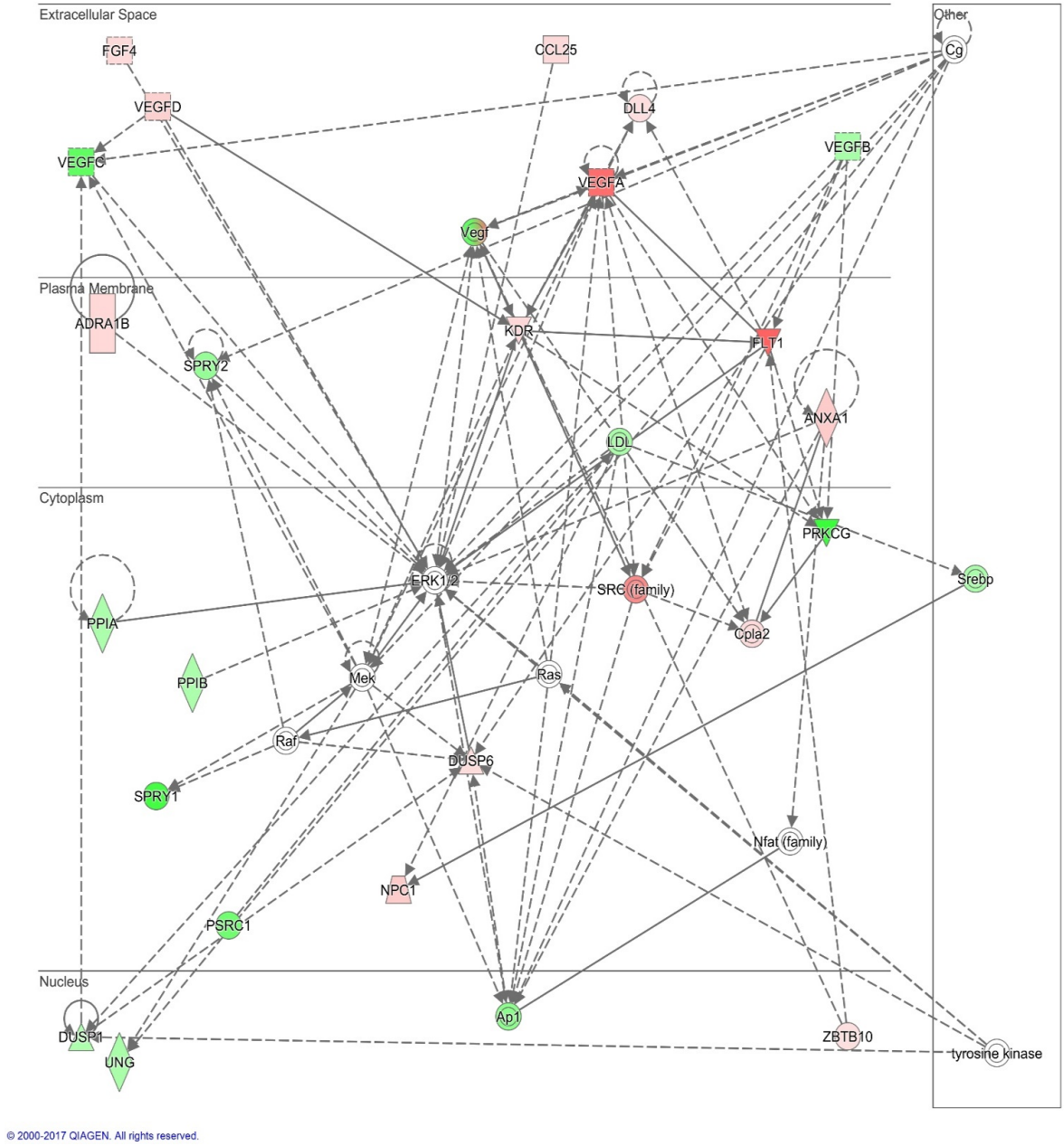
Network 10. Dermatological Diseases and Conditions, Organismal Injury and Abnormalities, Digestive System Development and Function.



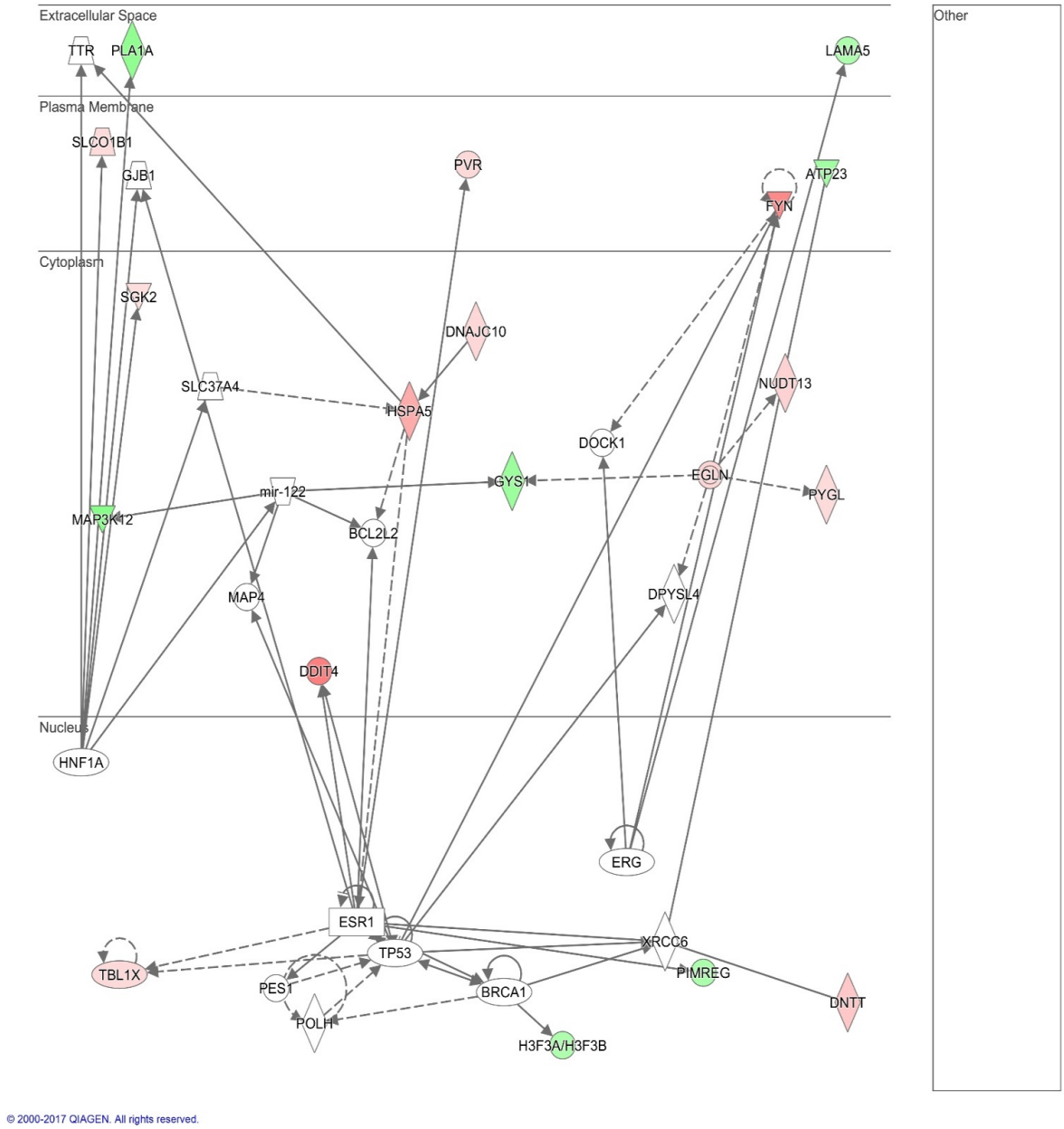
Network 11. Endocrine System Disorders, Gastrointestinal Disease, Metabolic Disease.



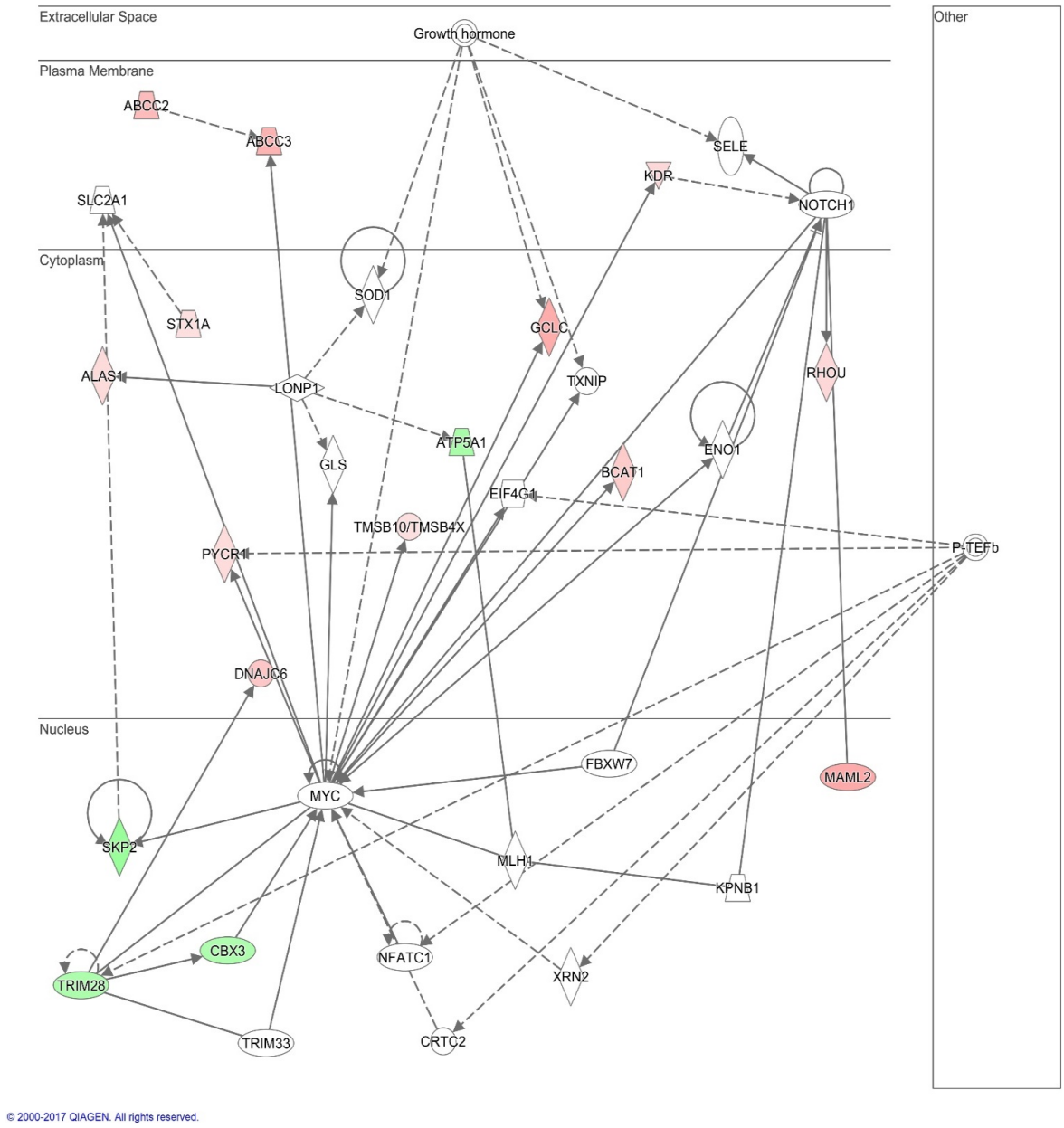
Network 12. Cancer, Hematological Disease, Organismal Injury and Abnormalities.



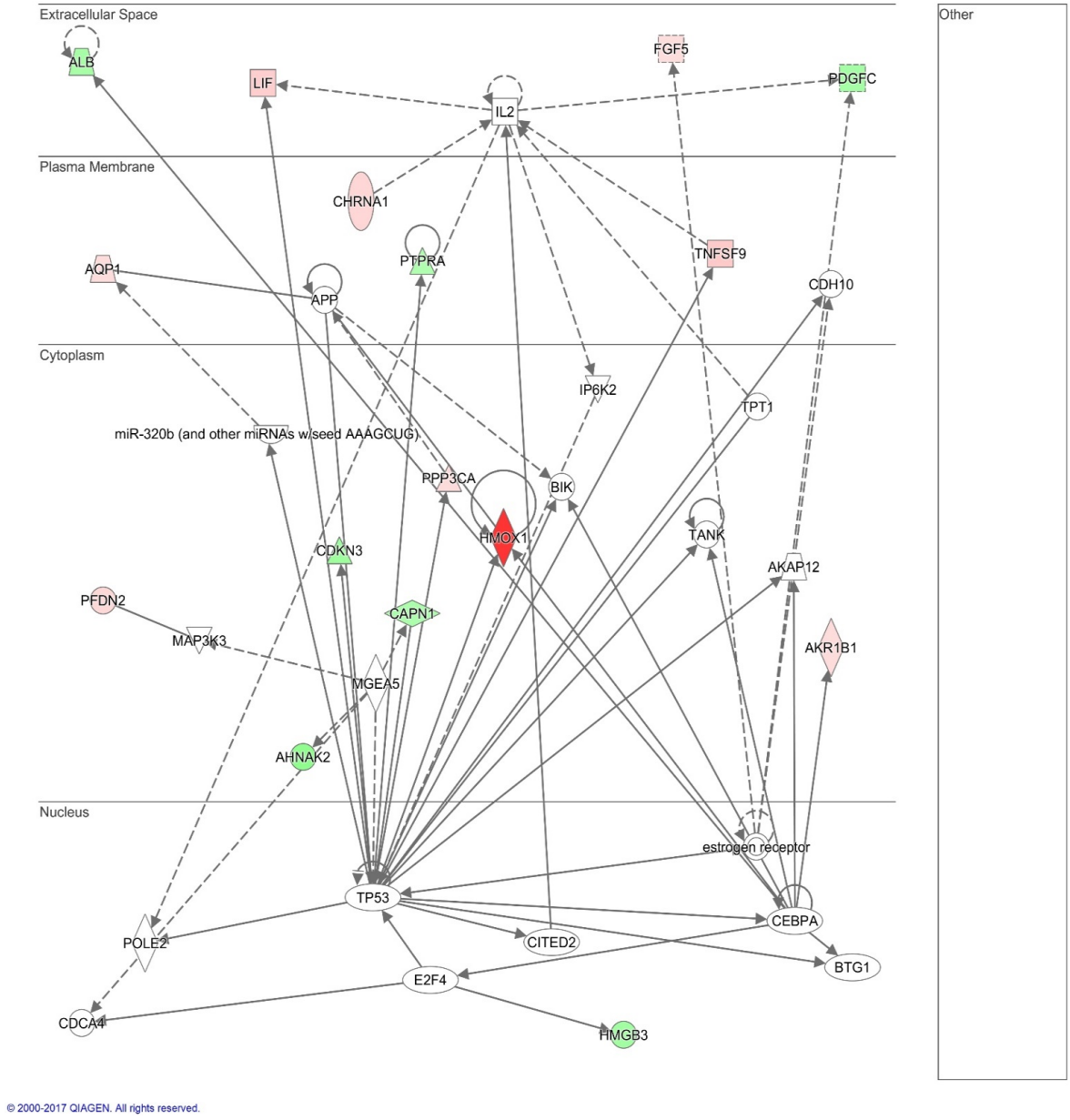
Network 13. Cardiovascular System Development and Function, Cell Cycle, Cell Morphology.



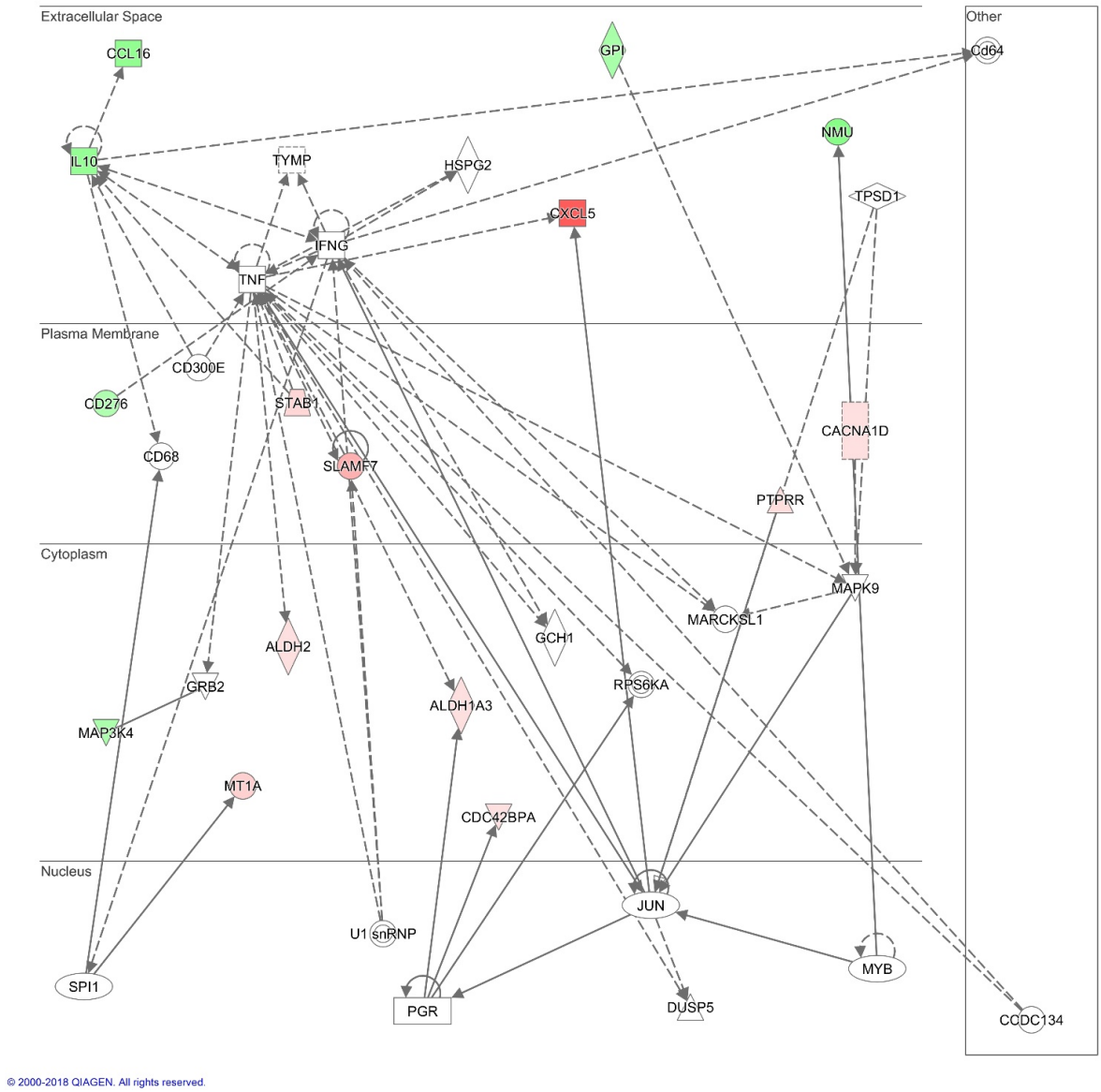
Network 14. Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function.



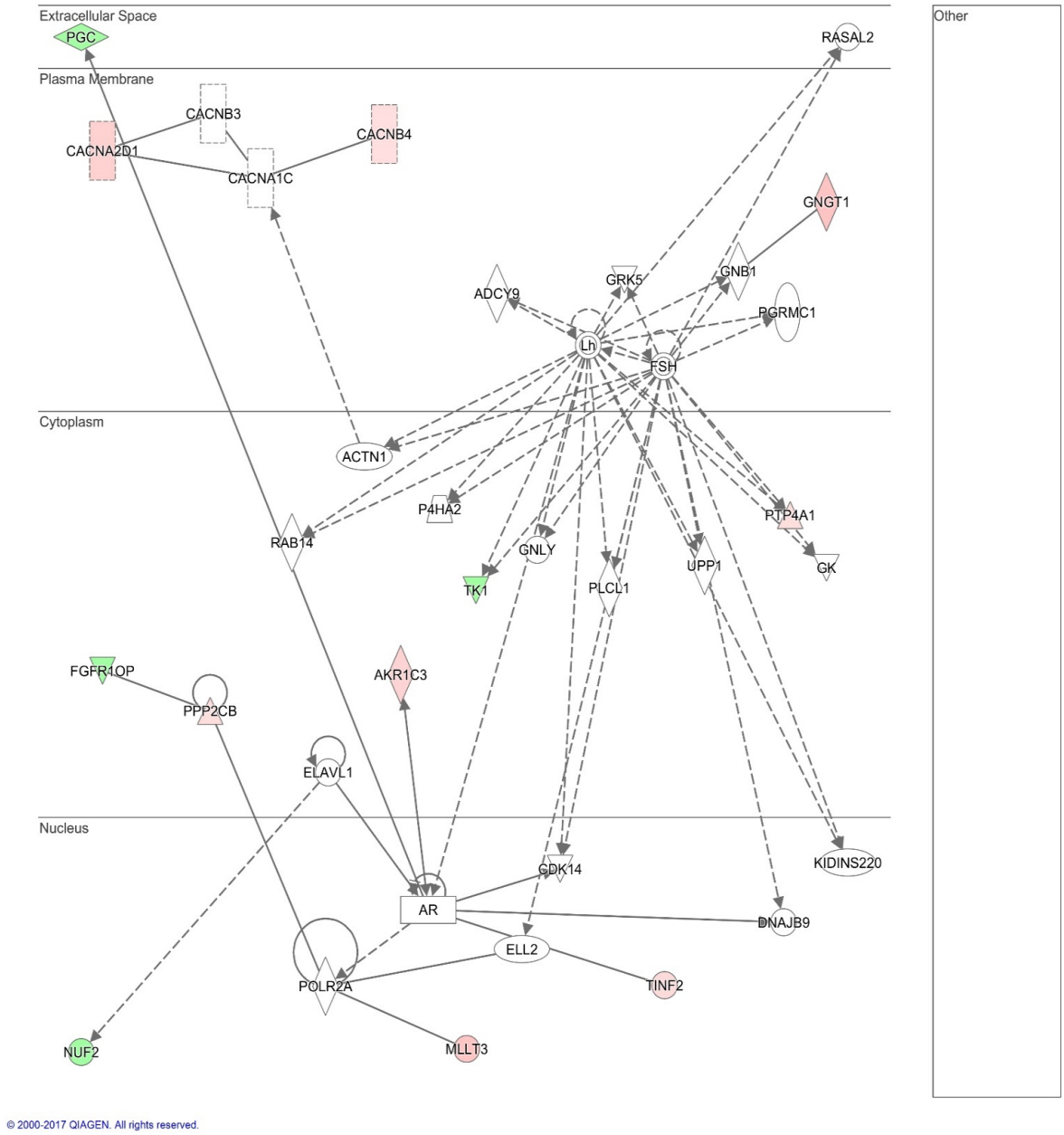
Network 15. Cancer, Hematological Disease, Immunological Disease.



Network 16. Cell Death and Survival, Inflammatory Disease, Inflammatory Response.



Network 17. Cellular Movement, Cellular Development, Cellular Growth and Proliferation.



Network 18. Cardiac Arteriopathy, Cardiovascular Disease, Organismal Injury and Abnormalities.