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Next generation sequencing and functional pathway analysis to understand the mechanism of action of copper-tolfenamic acid against pancreatic cancer cells

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Abstract

Anti-cancer activity of tolfenamic acid (TA) in preclinical models for pancreatic cancer (PaCa) is well established. Since the dosage for anti-cancer actions of TA is rather high, we recently demonstrated that IC₅₀ values of Copper-TA are 30–80% less than TA in 12 cancer cell lines. This study elucidates the underlying mechanisms of Copper-TA in PaCa cells. Control and Copper-TA (IC₅₀) treated PaCa cells were processed by next-generation sequencing (NGS) to determine differentially expressed genes using HTG EdgeSeq Oncology Biomarker panel. Ingenuity Pathway Analysis (IPA®) was used to identify functional significance of altered genes. The conformational studies for assessing the expression of key regulators and genes were conducted by Western blot and qPCR. IPA® identified several networks, regulators, as well as molecular and cellular functions associated with cancer. The top 5 molecular and cellular functions affected by Cu-TA treatment were cell death and survival, cellular development, cell growth and proliferation, cell cycle and cellular movement. The expression of top upstream regulators was confirmed by Western blot analysis while qPCR results of selected genes demonstrated that Copper-TA is

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Declarations of Competing interest
None.

efficacious at lower doses than TA. Results suggest that Copper-TA alters genes/key regulators associated with cancer and potentially serve as an effective anti-cancer agent.

Keywords

Pancreatic cancer; Apoptosis; Copper-tolfenamic acid; Next generation sequencing; Gene expression; Pathway analysis

1. 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 malignancies with poor prognosis [1,2]. The overall 5-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]. These cytotoxic therapies have a number of negative side effects such as vomiting, hair loss, low blood count and peripheral neuropathy [5]. Surgery is another treatment option; however, this approach is not feasible to more than 80% of patients due to late stage diagnosis [6,7]. 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 [8,9]. 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-tumor agents has gained tremendous popularity. NSAIDs work by inhibiting cyclooxygenase (COX) enzymes 1 and 2 [10]. Preclinical studies for NSAIDs have demonstrated its use as chemopreventive and chemotherapeutic drugs [11,12]. They have been shown to possess anti-cancer properties, such as inhibition of cancer cell growth and induction of apoptosis [13]. Another aspect that makes NSAIDs an attractive potential agent is that they also have lower toxicity in comparison to conventional chemotherapeutic drugs [14]. One of the NSAIDs of particular interest, is tolfenamic acid (TA) commonly sold in Europe as a generic medicine for migraine headaches. Many studies have been conducted to demonstrate TA's anti-neoplastic activity in multiple cancer models, including PaCa [15–17]. TA has been investigated not only because of its ability to modulate functional processes in cancer cells such as cell cycle and apoptosis, but also because of its low toxicity in non-malignant cells [18]. 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 [19]. The zinc finger transcription factors Sp1 and Sp3 play critical roles in the tumor formation and progression in PaCa [20]. These transcription factors regulate genes involved in many biological processes including cell growth and survival, cell cycle, and apoptosis. They also regulate survivin, a gene that inhibits apoptosis and also regulate cell

cycle [21]. We even used a mouse model to show that TA treatment sensitizes PaCa cells to radiation treatment by inhibiting survivin expression [22]. For these reasons, TA has been a drug of interest in cancer research to potentially use in combination therapies.

Thus far, the success of TA treatment in PaCa models appears 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. 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 [23,24]. 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 [25,26]. Their usefulness in combination treatments is due to their anti-cancer properties and limited toxicity [27–29]. 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 [30]. Recently, we demonstrated that TA prepared as a copper complex (Cu-TA) results in enhanced anti-proliferative activity in various models, including PaCa, when compared to TA [31]. Cu-TA inhibited PaCa cell proliferation and tumor growth in mouse xenograft model. In this study, 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 determine differentially expressed genes with Cu-TA treatment for pathway analysis to understand the functional significance of the altered gene expression. These results were compared to published work on TA to see if Cu-TA was 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.

2. Materials and methods

2.1. Cell lines and culture conditions

PaCa cells, MIA PaCa-2 (source: pancreatic epithelial cancer cells of tumor resected from 65-year-old Caucasian; hypotriploid cell line, 61 chromosomes) and PANC 1 2 (source: pancreatic epithelial cancer cells of tumor resected from 56-year-old Caucasian; hypotriploid cell line, cells with 63 or 61 chromosomes) were initially acquired from American Type Culture Collection (ATCC) (Manassas, VA) and authenticated using STR profiling by ATCC in July 2018 and found exact match for both cell lines. Cells were maintained in Dulbecco's Modified Eagle Medium media with high glucose (4500 mg/L) supplemented with fetal bovine serum and penicillin streptomycin as described before [32]. Cells were maintained in an incubator with optimal cell culture conditions (5% CO₂; 37 °C).

2.2. Reagents

Dimethyl sulfoxide (DMSO) and TA were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Cu-TA complex was synthesized using a previously established method [33]. A stock solution of Cu-TA was then made in DMSO with a concentration of 10 mM.

2.3. Next Generation Sequencing (NGS)

MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA using Cu-TA's IC₅₀ value (29 μ M) for 48 h. Cell lysates were collected and washed in PBS. Then 250 μ l 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 NGS assay uses quantitative nuclease protection (qNPA) to examine 2560 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 unhybridized 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 Supplementary data Figure S1.

2.4. Caspase 3/7 assay

PaCa cells were seeded in 96-well plates with each well containing 4000 cells in 50 μ l of media. 10 mM stock concentrations of each drug were diluted in 50 μ l 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 plated in a 96-well plate and then treated with DMSO (control), Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) 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 (BioTek) plate reader.

2.5. Ingenuity pathway analysis®

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: 43,605,602; Redwood City, CA) for expression analysis (Core Analysis module), and the program was used with its default settings.

2.6. Western blot

PaCa cells were treated with DMSO (control) or IC₅₀ dose of Cu-TA. 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), cleaved PARP (Cell Signaling Technology, Danvers, MA), survivin (R&D Systems, Minneapolis, MN), 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).

2.7. Quantitative Polymerase Chain Reaction (qPCR)

MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA (29 μ M) 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.

2.8. Statistical analysis

HTG Molecular Diagnostics at UT Southwestern Medical Center (Dallas, TX) analyzed the RNA sequencing results and provided 'fold-change' and 'significance (p value)' to identify the genes that were differentially expressed among control and Cu-TA treated samples. IPA® was used with its built-in statistical module, and $p < 0.05$ considered significant.

3. Results

The HTG EdgeSeq Oncology Biomarker panel consisting of 2560 genes was used in this study. After analyzing the sequencing data of differential 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.

3.1. Networks affected by Cu-TA treatment

The differentially expressed genes in PaCa cells with Cu-TA treatment found to be organized into a total of 18 networks (Table 1; Supplementary data Figures S2 to S19). These networks were associated with cancer and related diseases broadly and, also, implicating specific causative links (e.g., cell death/survival, immunological disease, organismal injury/abnormalities, cellular growth/proliferation), feature (morphology), and biological processes (system development/function, cell cycle, DNA replication, recombination/repair, cellular movement, inflammatory response, etc.). Fig. 1 shows a visual representation of a representative network merging several related networks into one picture to illustrate complexity. The dense lines indicate the overlapping connections and similarities between various networks. Upregulated and downregulated genes found by our NGS were colored in green and red, respectively.

3.2. Overlapping networks and a closer look at a selected network

Figs. 1 and 2A show the overlap of the 18 networks involved with Cu-TA treatments. Since, we observed induction of apoptosis and inhibition of anti-apoptotic markers with Cu-TA treatment, we selected network #7 (Fig. 2B) 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. Caspase 3/7, and c-PARP and survivin were evaluated. Cu-TA increased caspase 3/7 activity (Fig. 3A) and protein expression of c-PARP and down-regulated survivin (Fig. 3B).

3.3. Regulators involved with Cu-TA-induced gene alterations

In order to confirm the sequencing and IPA® results, the change in the protein expression of important regulators was determined by Western blot analysis for TP53, ErbB2, Sp1, and STAT3 (Fig. 4B). The protein expression (Western blot) results were correlated with both pathway analysis and NGS sequencing results (Fig. 4C). Consistent with gene expression, protein expression of TP53 was found to be increased with Cu-TA treatment while there was a downregulation in ErbB2, Sp1, and STAT3 expression.

IPA® analysis revealed the up-stream regulators that were 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), TGFBI (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) (Fig. 4A). Their *p*-value is also given along with their predicted activation status. The top networks and network functions that were affected were also presented in Table 1. Each network was 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.

3.4. Top associated molecular and cellular functions affected

Several molecular and cellular functions in the PaCa cells were influenced with the treatment. The top 5 functions are: cell death and survival, cellular development, cell growth and proliferation, cell cycle and cellular movement. Fig. 5A shows this list alongside with their corresponding *p*-values. The number of molecules affected (#Molecules column of the table) is also shown and is out of the 436 genes used as input for the IPA® software.

3.5. 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 [34]. DDIT3 is a transcription factor involved with activation of apoptosis during stress caused to the endoplasmic reticulum [35]. 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 [36]. A high expression of SKP2 is associated with metastasis and poor prognosis in PaCa patients [37]. 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 1). CENPF, DDIT3 and SKP2 were probed for qPCR analysis in MIA PaCa-2 cells treated with Cu-TA and

these results were then compared with a previous pathway analysis study using TA's IC₅₀ value [19] (Fig. 5B & C). 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.

4. 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 [5,38]. 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. Several investigations shown the potential of the small-molecule TA as an anti-cancer agent for a variety of different malignancies, including PaCa [15,22,32,39–42]. Recently, published work demonstrated that when TA is prepared as Cu-TA, it results in 30–80% enhancement of potency against cancer cell growth when compared to TA [31]. This study 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 cells (cardiomyocytes). In the current investigation, 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 (Table1; Supplementary data Figures S2 to S19). There were a large number of overlapping networks and molecular/cellular functions, showing that these networks are interdependent. 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 [43]. Mutated TP53 is one of the most common mutations found in human malignancies, including PaCa [44]. The MIA PaCa-2 cell line used in this study also has a mutation in TP53, affecting its activity [45]. 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 [46]. 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 [47]. 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 [48]. Upon dimerization and activation, ErbB2 regulates processes such as proliferation and migration [49]. ErbB2 overexpression has been found in PaCa and this expression results in a poorer prognosis [50]. As previously mentioned, Sp1 is a transcription factor found to be overexpressed and contribute to the progression of PaCa [51].

STAT3 is a transcription factor responsible for regulating several genes involving cell proliferation, survival, apoptosis, and invasion [52]. STAT3 has been found to be constitutively active in several malignancies, including PaCa and the hyperactivation of

STAT3 contributes to tumor progression and resistance to treatment [53,54]. 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 (Fig. 2B) 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 [21]. PARP is a DNA repair protein that becomes cleaved and, thus inactivated by effector caspases during apoptosis. Therefore, 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 MIA PaCa-2 cells.

Additionally, the top 5 molecular and cellular functions affected included cell death and survival, cellular development, cell growth and proliferation, cell cycle and cellular movement (Fig. 5A). 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 [19]. While, 50 μ M dose was used for TA treatment, only 29 μ M 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 (Fig. 5B & C) to previous study for TA (downregulation for CENPF and SKP2, upregulation for DDIT3). This suggests that TA and Cu-TA are perhaps working in a similar mechanism affecting cell cycle and apoptosis genes.

5. Conclusion

In conclusion, our NGS sequencing and pathway analysis results provide the first insight into networks, pathways, and biological and molecular functions affected by Cu-TA treatment in PaCa cells. Apoptosis, cell cycle, 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 (Figs. 6). As presented in the schematic (Fig. 6), the overall analyses of all results (NGS, 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 published studies further confirm Cu-TA's potential as an anti-tumor agent for PaCa. Furthermore, these outcomes of the study may also be valuable for a better comprehension of pancreatic tumor biology and perhaps for the identification of novel potential therapeutic targets against PaCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Merged Networks (Core of #1 Overlapping Networks). 18 networks were found to be correlated with the differentially gene expression induced by Cu-TA treatment. This shows the core of #1 overlapping networks merged into a single network.

Fig. 2. Overlapping Networks and Selection of Network #7 for Further Analysis. The 18 networks (A) are displayed and show that they have overlapping functions. Network #7 (B) was selected to show the molecules involved in this particular network. The genes in green indicate downregulation, while red signifies upregulation by TA treatment. The intensity of the color correlates to the degree of down- or upregulation.

Fig. 3.

Confirmation of Critical Markers of Network#7. Caspase 3/7 activity (A) was measured using Capase-Glo kit and the results were normalized to cell viability. Bar diagram depicts the mean $\pm$ SEM of triplicate samples. The expression of critical markers primarily associated with cancer cell growth and apoptosis, c-PARP and survivin was determined by Western blot analysis and representative bands were given in Figure.

Fig. 4.

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 with *p*-value of < 0.001. (B) MIA PaCa-2 cells were treated with DMSO (control) or Cu-TA (29 μ M) 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. Table (C) shows HTG sequencing results and gives the up or

downregulation fold change of the confirmed regulators. Downregulation fold change is given in green and upregulation is shown in red.

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Fig. 5. Top Diseases and Bio Functions (Molecular and Cellular Functions) Revealed by Cu-TA as Analyzed by Ingenuity Pathway Analysis®. Changes in the mRNA expression of critical genes. (A) List of top diseases and bio functions and #molecules indicates how many molecules in that disease/biological function are affected. The *p*-value is < 0.001 for all of the diseases and functions listed. (B) MIA PaCa-2 and PANC 1 cells were treated with DMSO (control) or Cu-TA (IC₅₀ dose) for 48 h. mRNA expression levels of CENPF, DDIT3 and SKP2 were then probed for while GAPDH was used as a loading control. (C) Data

shown gives the fold change of genes normalized to the control. This data was then compared with the results from a previous pathway analysis study using TA's IC_{50} value.

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Fig. 6. Cu-TA's Biological Targets in MIA PaCa-2 Cells. The schematic diagram showing Cu-TA's mechanism of action is based on 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 downregulation while green indicates upregulation. The outline color of the text indicates the method used.

Table 1

Top Networks: ID Associated Network Functions. Gene expression correlation with the top networks affected after 48 h of Cu-TA treatment in MIA PaCa-2 cells. The score indicates how many molecules are affected out of 436 significantly affected genes used as input.

	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