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Myrna Hurtado

Umesh T. Sankpal

Aboubacar Kaba

Shahela Mahammad

Jaya Chhabra
Old Dominion University

See next page for additional authors

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Authors

Myrna Hurtado, Umesh T. Sankpal, Aboubacar Kaba, Shahela Mahammad, Jaya Chhabra, Deondra T. Brown, Raj K. Gurung, Alvin A. Holder, Jamboor K. Vishwanatha, and Riyaz Basha

Original Paper

Novel Survivin Inhibitor for Suppressing Pancreatic Cancer Cells Growth via Downregulating Sp1 and Sp3 Transcription Factors

Myrna Hurtado^a Umesh T. Sankpal^b Aboubacar Kaba^a Shahela Mahammad^b
Jaya Chhabra^c Deondra T. Brown^c Raj K. Gurung^c Alvin A. Holder^c
Jamboor K. Vishwanatha^a Riyaz Basha^{a,b}

^aGraduate School of Biomedical Sciences, UNT Health Science Center, Fort Worth, ^bTexas College of Osteopathic Medicine, UNT Health Science Center, Fort Worth, ^cDepartment of Chemistry and Biochemistry Old Dominion University, Norfolk, USA

Key Words

Pancreatic cancer • Sp1 • Sp3 • Survivin • Copper-tolfenamic acid

Abstract

Background/Aims: Targeting survivin, an anti-apoptotic protein and mitotic regulator, is considered as an effective therapeutic option for pancreatic cancer (PaCa). Tolfenamic acid (TA) showed anti-cancer activity in pre-clinical studies. A recent discovery demonstrated a copper(II) complex of TA (Cu-TA) can result in higher activity. In this study, the ability of Cu-TA to inhibit survivin and its transcription factors, Specificity protein (Sp) 1 and 3 in PaCa cell lines and tumor growth in mouse xenograft model were evaluated. **Methods:** Cell growth inhibition was measured in MIA PaCa-2 and Panc1 cells for 2 days using CellTiter-Glo kit. Sp1, Sp3 and survivin expression (by Western blot and qPCR), apoptotic cells and cell cycle phase distribution (by flow cytometry) were evaluated. A pilot study was performed using athymic nude mice [treated with vehicle/Cu-TA (25 or 50 mg/kg) 3 times/week for 4 weeks. **Results:** The IC₅₀ value for Cu-TA was about half than TA. Both agents repressed the protein expression of Sp1/Sp3/survivin, Cu-TA was more effective than TA. Especially effect on survivin inhibition was 5.2 (MIA PaCa-2) or 6.4 (Panc1) fold higher and mRNA expression of only survivin was decreased. Apoptotic cells increased with Cu-TA treatment in both cell lines, while Panc1 showed both effect on apoptosis and cell cycle (G₂/M) arrest. Cu-TA decreased the tumor growth in mouse xenografts (25 mg/kg: 48%; 50 mg/kg: 68%). Additionally, there was no change observed in mice body weights, indicating no overt toxicity was occurring. **Conclusion:** These results show that Cu-TA can serve as an effective survivin inhibitor for inhibiting PaCa cell growth.

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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]. 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]. Konduri et al. (2009) also demonstrated that TA 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]. They can regulate their target gene transcription by binding to GC-rich elements and promoter sequences. Sp1 overexpression is associated with tumor formation and progression, metastasis, and angiogenesis [25]. Therefore, it has even been suggested as a potential biomarker to detect a class of advanced pancreatic ductal adenocarcinoma [26].

One gene of particular interest in PaCa regulated by these Sp1 and Sp3 is survivin [27]. 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 μ M), strategies to enhance its efficacy 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 [28-34]. Recently, a copper (II)-Tolfenamic acid has been synthesized and published [35]. We have recently evaluated the anti-proliferative activity of Cu(II)- containing complex of TA (Cu-TA) Cu-TA using twelve human cancer cells lines representing six cancers [36]. This screening study found that TA was chemically and biologically stable and demonstrated a higher efficacy in comparison to TA. Additionally, there was no overt toxicity observed in the

Cu-TA treated cardiomyocytes. In this study, we assessed the anti-cancer efficacy of Cu-TA in detail using human PaCa cell lines. The results of this study showed higher anti-cancer activity of Cu-TA which was accompanied by a substantially higher increase in the inhibition of survivin when compared to TA.

Materials and Methods

Cell lines and Culture Conditions

Two human pancreatic ductal adenocarcinoma cell lines, MIA PaCa-2 and Panc1 were obtained American Type Culture Collection (ATCC) and authenticated using STR profiling by ATCC in July, 2018. Both cell lines resulted in 100% match with the ATCC database. Both cell lines were grown and cultivated in Dulbecco's Modified Eagle Medium (DMEM) media with high glucose (4500 mg/L) supplemented with 5% fetal bovine serum and 1% penicillin streptomycin. Cells were maintained in an incubator at 37 °C with 5% CO₂.

Preparation of Stock Concentrations

Tolfenamic acid was purchased from Sigma-Aldrich Corporation and a 10 mM stock solution was prepared in dimethyl sulfoxide (DMSO). Commercially available copper(II) chloride (CuCl₂) and 2, 2'-bipyridine (bpy) were used as controls. The copper(II)-tolfenamic acid (Cu-TA) complex was synthesized using an established method [35]. A 10 mM stock solution of CuCl₂, BPY, and Cu-TA were all prepared in DMSO.

Cell Viability

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. Each treatment was carried out in triplicates with increasing concentrations of DMSO (vehicle), TA (10, 25, 50, and 100 µM), or Cu-TA (10, 25, and 50 µM). Cells were then incubated with CellTiter-Glo reagent 24 and 48 h post-treatment and incubated in the dark for 25 min. Cell viability was then measured by detecting luminescence using Synergy HT (BioTek) plate reader. For all subsequent experiments the concentration of Cu-TA's IC₅₀ value and equimolar TA were used. To test the cytotoxic effect of the components used in the synthesis of Cu-TA (CuCl₂ and BPY), MIA PaCa-2 and Panc1 cells were treated with DMSO, CuCl₂ or BPY using equimolar concentration of Cu-TA's IC₅₀ value (MIA PaCa-2: 29 µM; Panc1: 27 µM). Cell viability was measured as explained above.

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 plated in a 96-well plate and then treated with DMSO (control), 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 (BioTek) plate reader.

Western Blot

MIA PaCa-2 and Panc1 cells were plated in 10 cm petri dishes containing 750,000 cells in 10 ml of media. Cell 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 plated in 10 cm petri dishes containing 750,000 cells in 10 ml of media. Cells were treated with DMSO (control), Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) or equimolar TA for 48 h. At the time of collection, total RNA was extracted from treated cells using TRIzol (Invitrogen). Total RNA was then converted into single-stranded cDNA using Superscript III (Invitrogen). After cDNA amplification, genes were probed for using TaqMan Gene Expression Assays (Applied Biosystems) 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.

Flow cytometry

PE Annexin V Staining: Apoptotic cell populations were detected using PE Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were plated in 6-well plates containing 2,000 cells in 2 ml of media. Cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) 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 were measured via FC500 flow cytometer.

Cell Cycle Arrest

MIA PaCa-2 and Panc1 cells were plated in 6-well plates containing 2,000 cells in 2 ml of media. Cells were treated with DMSO (control), Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) 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 °C 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 using FC500 Flow Cytometer.

Mouse Model

Athymic nu/nu nude mice were obtained from Charles River (Wilmington, MA). Mice were subcutaneously injected with 6×10^6 MIA PaCa-2 cells and the tumors were allowed to grow for 10 days ($\sim 100\text{mm}^3$ in diameter) before starting treatments. There were 3 treatment groups [vehicle (corn oil), Cu-TA 25 mg/kg or 50 mg/kg] with 4 mice in each group (half male and half female). Mice were treated 3 times/week for 4 weeks through oral gavage. Mouse body weights were recorded 3 times/week as well. At the end of 4 weeks, the mice were euthanized by CO₂ asphyxiation and their tumors were harvested. The tumor volume and weights were measured and the averages in each group were calculated (n=6). The procedures are approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center (protocol# IACUC-2017-0040).

Statistical Analysis

Data were analyzed using one-way ANOVA. Results were expressed as mean \pm SD. Significance between treated and untreated cells were evaluated, and a *p* value < 0.05 was considered as significant.

Results

TA and Cu-TA Inhibit PaCa Cell Growth

MIA PaCa-2 and Panc1 cells were treated with increasing concentrations of DMSO, TA and Cu-TA to evaluate the anti-proliferative activity and determine the optimal dosage for Cu-TA. Both TA and Cu-TA decreased cell viability in a dose and time dependent manner in both MIA PaCa-2 (Fig. 1A) and Panc1 cells (Fig. 1B). However, Cu-TA was found to be more effective than TA for inhibiting PaCa cell growth and at lower doses. The IC₅₀ value for Cu-TA at 48 h post-treatment (Fig. 1C) was found to be half (MIA PaCa-2: 29.32 μ M; Panc1: 26.65 μ M) that of TA's IC₅₀ value (MIA PaCa-2: 57.20 μ M; Panc1: 61.76 μ M). At 24 h, there was less than 50%

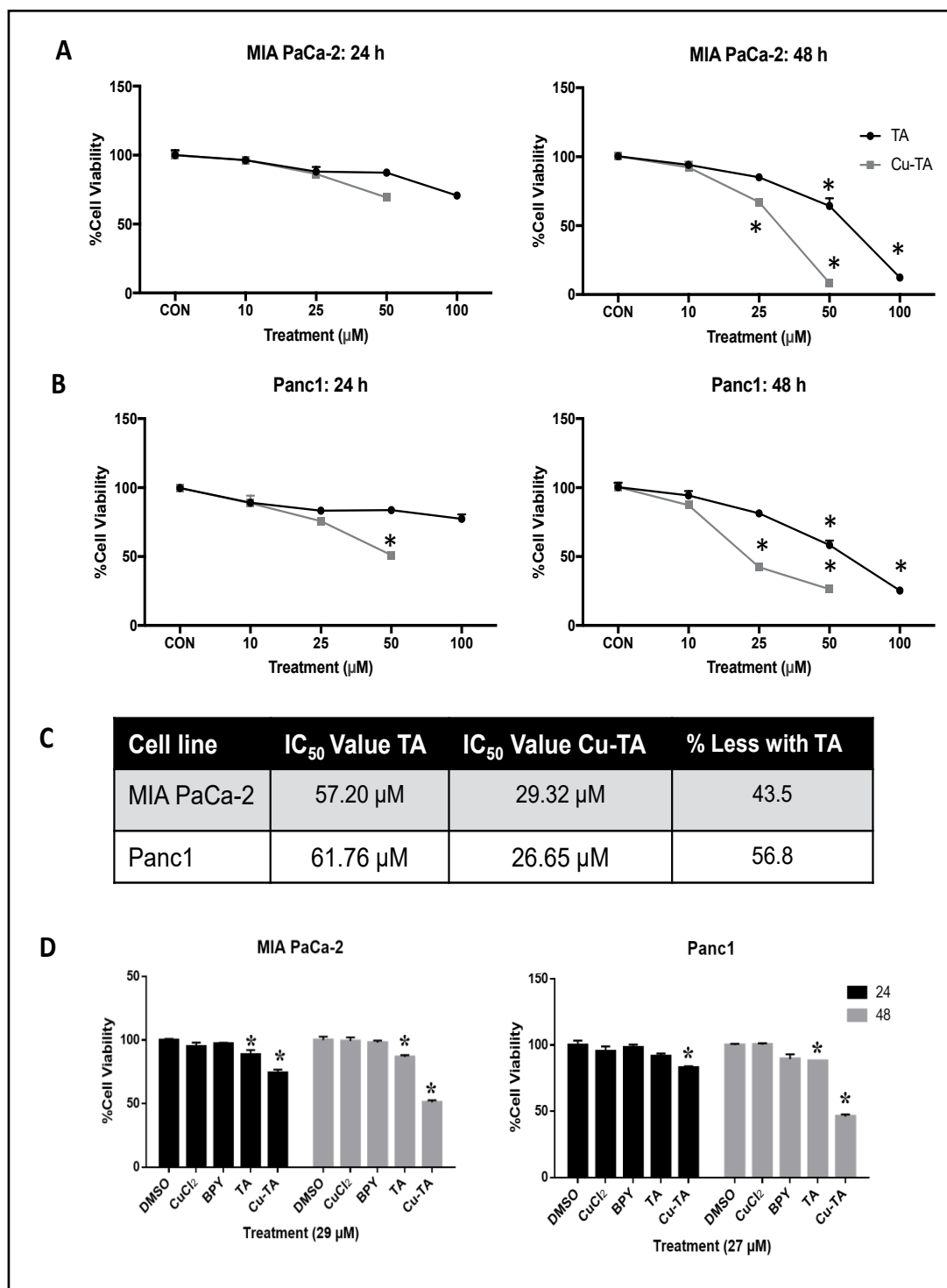


Fig. 1. Anti-proliferative effect of TA or Cu-TA against PaCa cells. MIA PaCa-2 (A) and Panc1 (B) cells were treated with DMSO or Cu-TA (5–50 μM) or TA (5–100 μM) and cell viability was measured after 24 and 48 h. Lines represent % viable cells (mean \pm SD) normalized to control. All treatments were done in triplicate. (C) IC₅₀ values for both cell lines at 48 h for TA and Cu-TA. (D) The effect of CuCl₂ and BPY on cell viability in MIA PaCa-2 and Panc1 cells was measured 24 and 48 h post-treatment. Bars represent % viable cells (mean \pm SD) normalized to control. All treatments were done in triplicate, and the bars marked with “*” are significantly different from corresponding control ($p < 0.0001$).

cell growth inhibition for both cell lines. Therefore, the IC_{50} values were calculated only at 48 h post-treatment and found that Cu-TA's IC_{50} values were 49% and 57% less (respectively in MIA PaCa-2 and Panc1) when compared to TA. Additionally, neither $CuCl_2$ nor BPY had any effect on cell viability (Fig. 1D).

Cu-TA Downregulates Sp1, Sp3 and Survivin Protein Expression

The effect of Cu-TA on Sp1, Sp3 and survivin protein expression was assessed via Western blot. There was a significant inhibition of Sp1, Sp3 and survivin protein expression with the Cu-TA 48 h treatment in both cell lines (Fig. 2A&B). TA is 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.

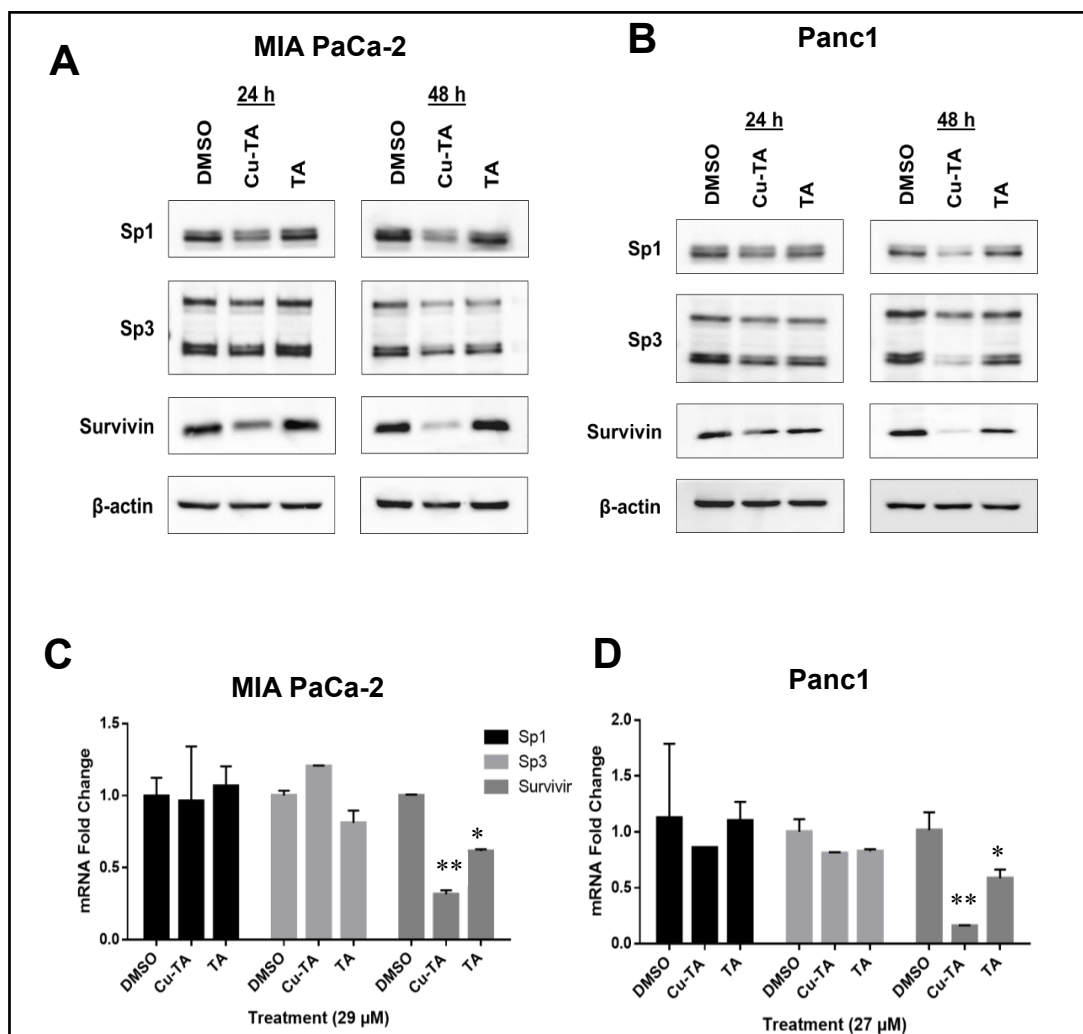


Fig. 2. Effect of Cu-TA on Sp1, Sp3 and survivin expression in PaCa cells. (A&B) MIA PaCa-2 and Panc1 cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) or equimolar TA for 24 and 48 h. Protein extracts from cell lysates were then prepared and the expression of Sp1, Sp3, survivin, and β -actin (loading control) was determined by Western blot analysis. All experiments were conducted with 3 sets of samples and the representative gels are shown in the Figure. (C&D) MIA PaCa-2 and Panc1 cells were treated with DMSO or Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) or equimolar 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 \pm SD) normalized to control. All treatments were done in triplicates, and the bars marked with '*' are significantly different from corresponding control ($P < 0.001$).

TA and Cu-TA Lower Survivin mRNA Expression Levels While Sp1 and Sp3 Remains Unaltered

Quantitative PCR results demonstrate that TA and Cu-TA treatments significantly lower survivin mRNA expression levels in both cell lines (Fig. 2C&D). This indicates that survivin is effected at the transcriptional level. However, Sp1 and Sp3 mRNA expression levels remain relatively unchanged in both cell lines.

Cu-TA Increases Apoptotic Markers and Apoptotic Cell Populations

Cells were treated with Cu-TA or TA and their effect on effector caspase 3/7 activity, protein expression of c-PARP and apoptotic cell populations were analyzed. Cu-TA treated cells had a significant increase in effector caspase 3/7 activity and this correlated with an increase in c-PARP expression (Fig. 3). A significant increase in apoptotic cell populations was also observed for the Cu-TA treatment (Fig. 4).

Cu-TA Causes G2/M Phase Arrest in Panc1 Cells

Cu-TA's effect on cell cycle phase distribution was determined by flow cytometric analysis. Cu-TA had no effect on MIA PaCa-2 cells (Fig. 5A), while there was an increase in G2/M phase in Panc1 cells (Fig. 5B; Fig. 6A) at 48 h. Confirmation of G2/M phase arrest was done by examining protein expression of G2/M markers, cyclin B1 and cyclin A (Fig. 6B).

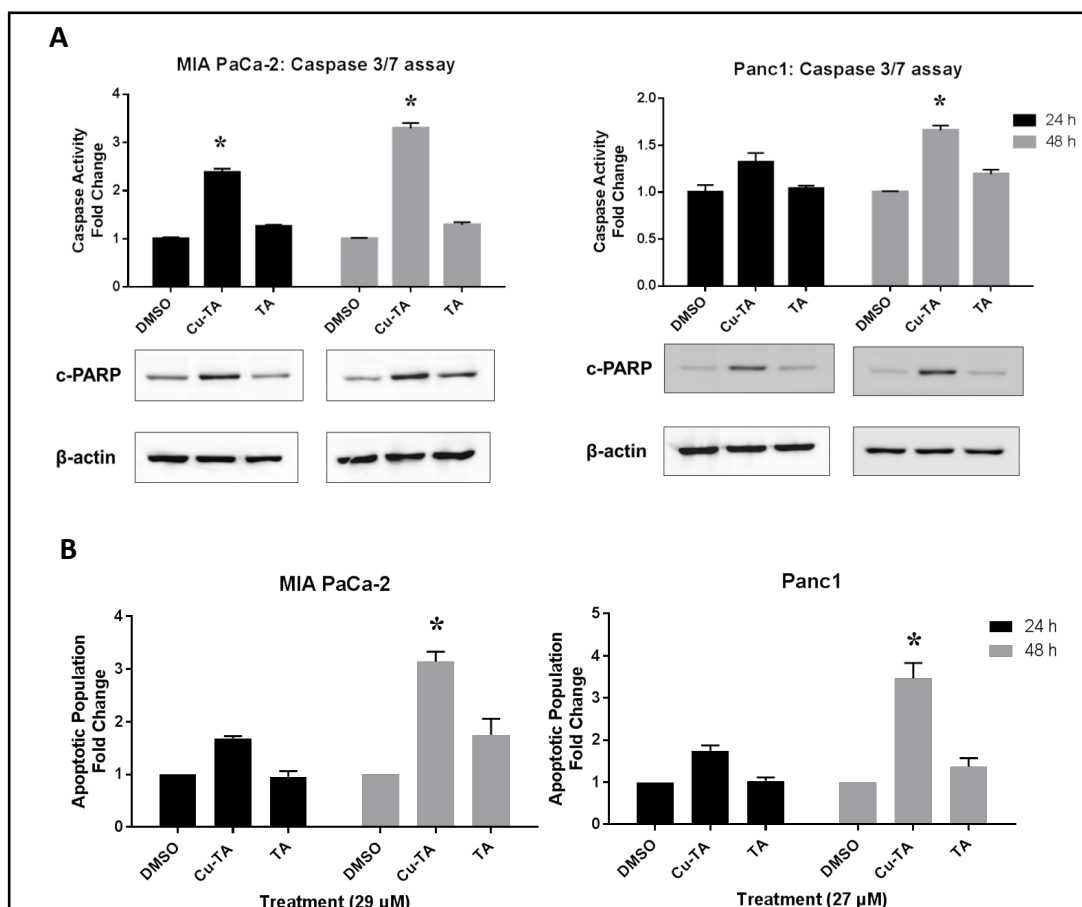


Fig. 3. Cu-TA upregulates effector caspase activity, cleaved PARP expression, and apoptotic cell populations in PaCa cells. (A) MIA PaCa-2 and Panc1 cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) or equimolar TA. Caspase 3/7 activity and c-PARP protein expression was determined at 24 and 48 h post-treatment. (B) shows the fold change of apoptotic cell populations in MIA PaCa-2 and Panc1. Bars represent mean \pm SD. All treatments were done in triplicates, and the bars marked with '*' are significantly different from corresponding control ($p < 0.001$).

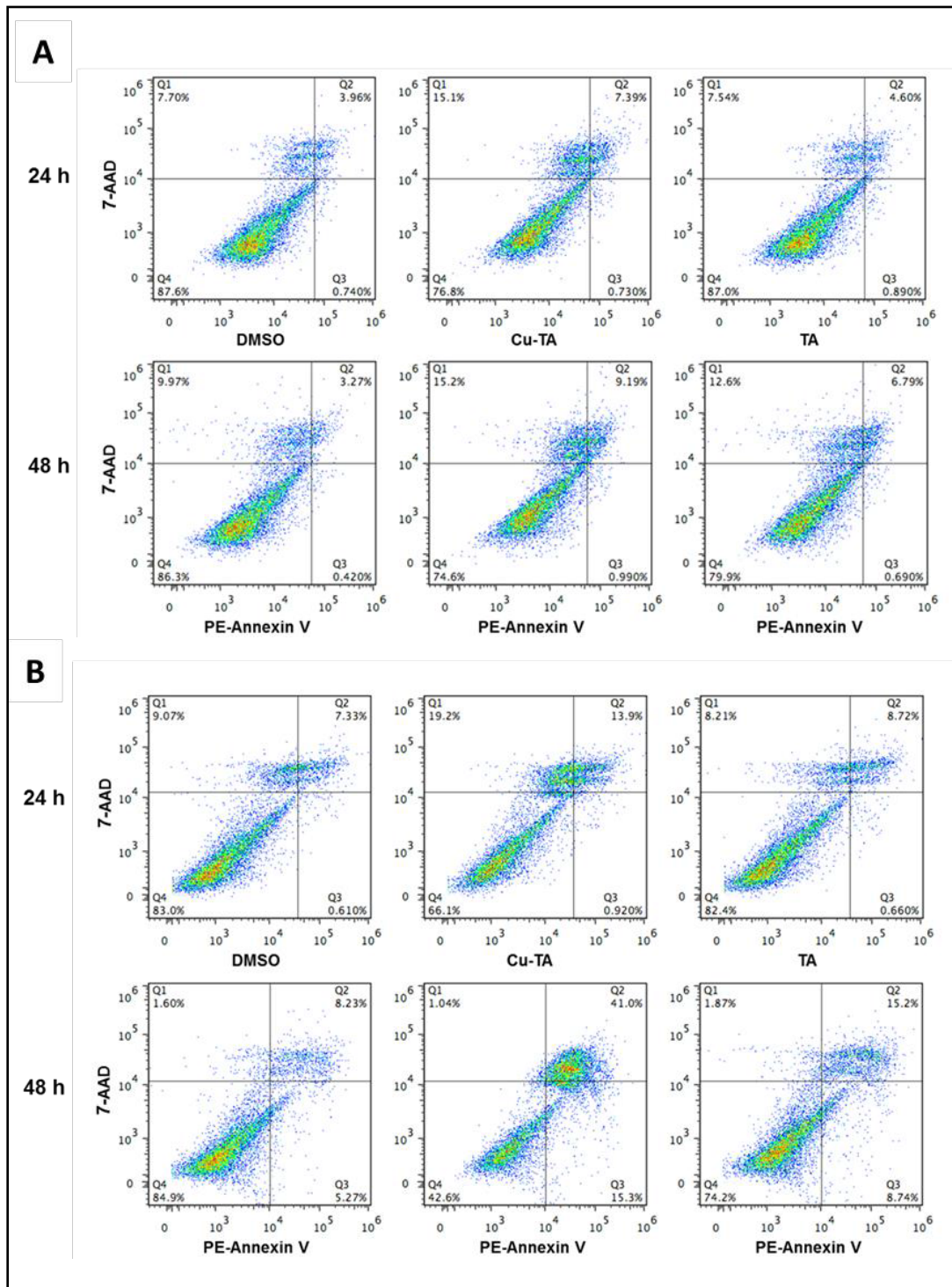


Fig. 4. Cu-TA increases apoptotic cell populations in PaCa cells. (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) or equimolar TA. Apoptotic cell populations were assessed using PE Annexin V Staining post 24 and 48 h treatment.

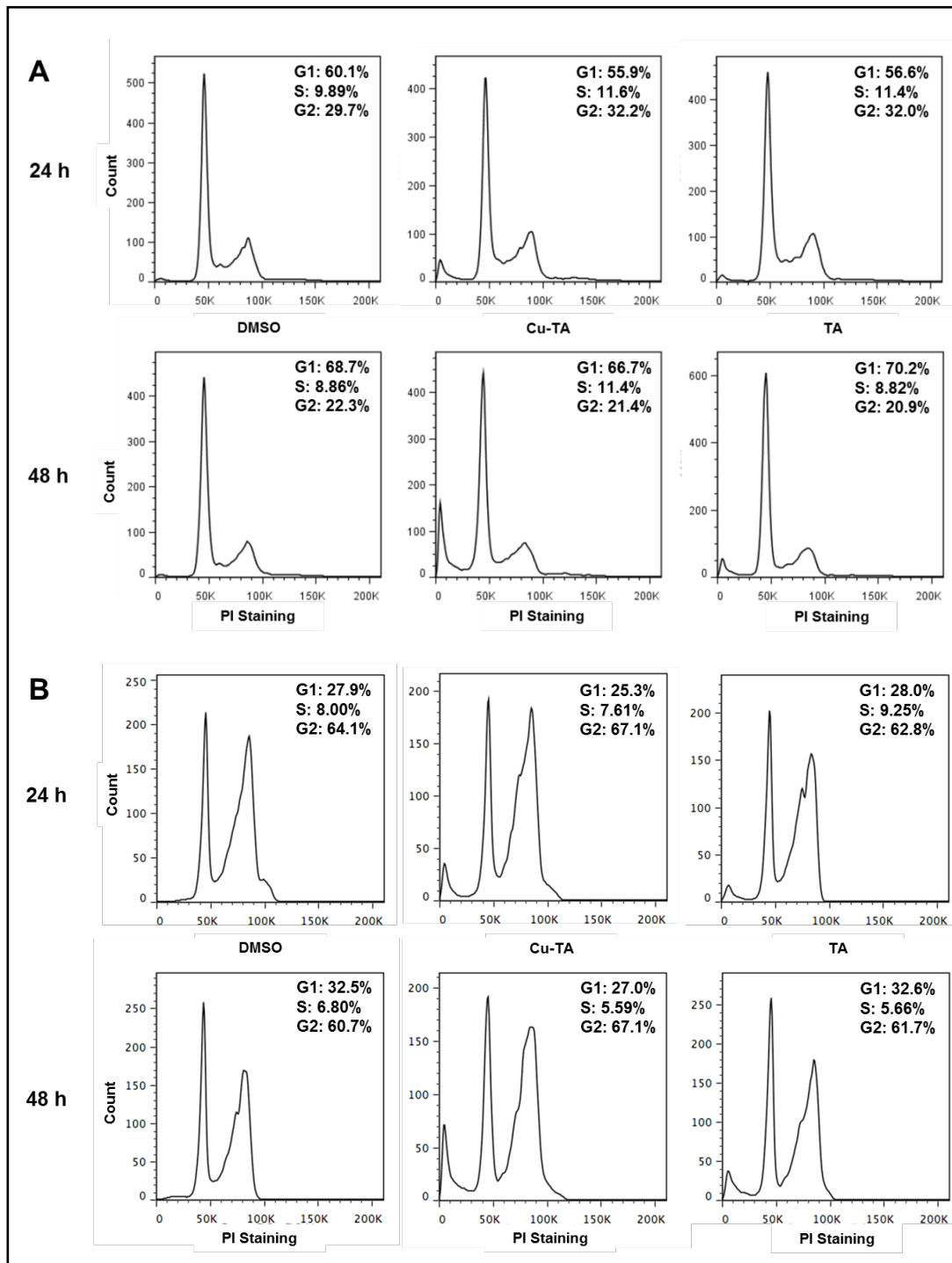
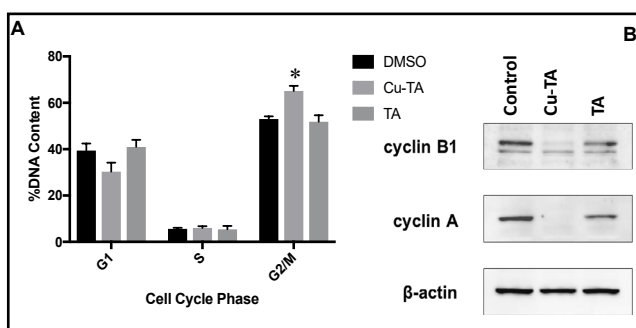


Fig. 5. Cu-TA causes G₂/M phase arrest in Panc1 cells. (A) MIA PaCa-2 and (B) Panc1 cells were treated with DMSO, Cu-TA (MIA PaCa-2: 29 μ M; Panc1: 27 μ M) or equimolar TA for 24 and 48 h post-treatment. Cells were collected and then incubated with propidium iodide DNA staining. Samples were then analyzed via flow cytometry. Peaks indicate respective phase (G₁, S, G₂/M) of the cell cycle.

Fig. 6. Confirmation of G₂/M phase arrest in Panc1 cells. (A) Panc1 cells were treated with DMSO, Cu-TA (27 μM) or equimolar TA for 48 h. 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 Panc1 cell lysates were then prepared and the expression of G₂/M phase markers cyclin B1, cyclin A and β-actin was determined by Western blot analysis.



Cu-TA Dose Dependently Inhibits Tumor Growth in PaCa Mouse Model

Both Cu-TA treatment groups caused a decrease in tumor weights and volume which appears to be a dose dependent response. The Cu-TA 25mg/kg group had a 48% decrease while the 50 mg/kg group had a 68% decrease in tumor weight (Fig. 7A). Tumor volume also showed similar trend showing 39% (25 mg/kg group) and 73% decrease (50 mg/kg group) decrease when compared to control group (Fig. 7B). In addition, there was a decrease in tumor vasculature as pictured (insert) in Fig. 7A. There was no change in mice body weights (Fig. 7C), indicating no overt toxicity.

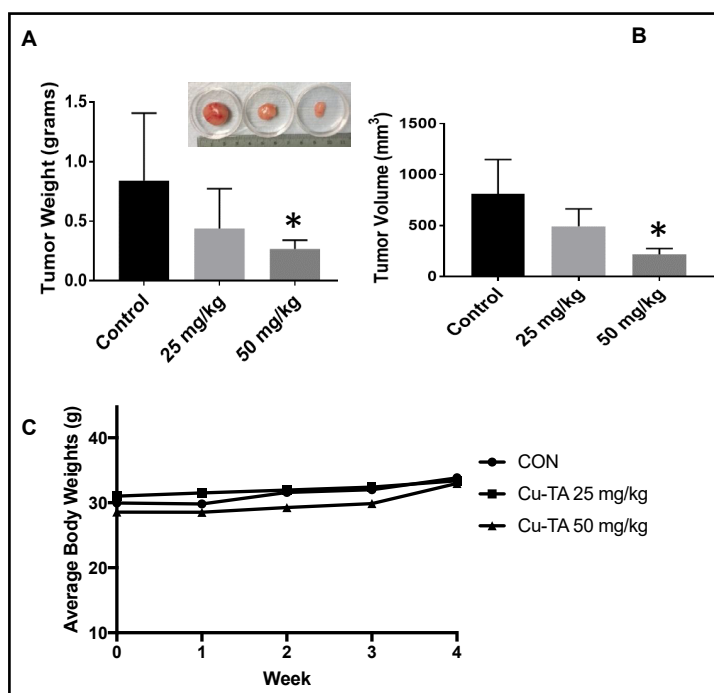


Fig. 7. Cu-TA inhibits PaCa tumor growth in mouse: Athymic nude mice were injected with 6 million MIA PaCa-2 cells and were treated with either vehicle (corn oil), Cu-TA 25 mg/kg or 50 mg/kg 3 times/week for 4 weeks. Mean (±SE) of (A) tumor weight and (B) tumor volume (representative tumor pictures in the insert) of the mice treated with vehicle or 25 mg/kg of Cu-TA or 50 mg/kg of Cu-TA (n=6 per group). (C) The body weights were measured each week and the weight (mean±SD) of animals were presented.

Discussion

The limited treatment options and therapy effectiveness pose a crucial need for novel treatments for PaCa. for their transformation and subsequent progression and resistance to radiation/chemotherapy [37]. Resistance to current therapeutic options is a major barrier in PaCa treatment. Therefore, an effective approach for treatment could be targeting the molecular mechanisms that drive tumorigenesis and resistance. In this study, we were particularly interested in survivin because of its known role in poor prognosis and resistance to treatment in PaCa. Due to morbidity associated with standard care, it is not plausible to increase the doses when the patients show poor response, thereby treatment of such patients is becoming even more challenging. Therefore, the strategies to identify less toxic agents to induce sensitivity in cancer cells and the therapeutic efficacy is our top priority. There have been an increasing

number of studies using NSAIDs as chemopreventive and chemotherapeutic agents [38], because NSAIDs are not only effective, but also less toxic in comparison to conventional chemotherapy drugs [39]. Several strategies including small molecules for inhibiting cell growth pathways have been tested [40, 41]. NSAIDs are small molecules that typically work by inhibiting cyclooxygenase (COX) enzymes and thereby modulating prostaglandins [42]. However, there are limitations to the use of NSAIDs for cancer therapy especially the issues associated with long-term use [43]. Therefore, there has been rising interest in finding NSAIDs that work independent of COX enzyme inhibition.

Our laboratory and others have demonstrated the effectiveness of TA (NSAID) for inhibiting PaCa cell growth in cancer models [20, 24, 44]. In this study, the cell viability results demonstrated that Cu-TA's anti-proliferative effect in PaCa cells is much more effective resulting the IC_{50} value about 50% less than TA in both cell lines (Fig. 1A&B). Furthermore, neither $CuCl_2$ nor BPY had any significant effect on cell growth in either cell line at 24 and 48 h post-treatment (Fig. 1C). This demonstrates that the decrease in cell viability is specific to the Cu-TA compound and not to any single agent alone. Protein analysis showed that Cu-TA treatment caused downregulation of Sp1, Sp3 and survivin (Fig. 2A&B). To investigate if this downregulation was due to the targets being effected on a transcriptional level or post-translationally, mRNA levels were examined. Survivin was found to be significantly downregulated on a transcriptional level while both Sp1 and Sp3 levels were comparatively unaffected (Fig. 2C&D) suggesting that significant decrease of survivin mRNA levels could be due to a lack of Sp1- and Sp3-dependent transcription [27]. These results correspond with previous TA data; TA is known to effect Sp1 and Sp3 post-translationally, through proteasome-dependent degradation [24]. This suggests that Cu-TA perhaps work in a similar mechanism to TA.

To investigate the cause of growth inhibitory response seen in the Cu-TA treatment, the apoptotic markers, caspase 3/7 and c-PARP and the number of apoptotic cells were evaluated. Thus, the increased caspase 3/7 activity and protein expression of c-PARP in the Cu-TA treatment suggests that the activation of apoptosis is occurring (Fig. 3A). Annexin-V has a high affinity for PS, thus, when linked to the fluorochrome phycoerythrin (PE), it is possible to detect apoptotic cell populations via flow cytometry [45]. Annexin-V flow cytometric results showed Cu-TA significantly increased apoptotic cell populations (Fig. 3A; Fig. 4), correlating with the increased caspase activity and cleaved PARP expression (Fig. 3A).

Cell cycle phase distribution was also examined. The Cu-TA treatment in Panc1 cells revealed an increase in G2/M phase cells compared to the control, indicating cell cycle arrest (Fig. 5B; Fig. 6A). Cell cycle is a highly regulated process involving a series of events that leads to faithful replication of cells. The transition and progression of cell cycle phase depends on differential expression of various cyclins [46]. Cyclins form complexes with their respective cyclin-dependent kinases (CDKs) which can then regulate transcription of target genes [47]. The expression of cyclin A and B1 were evaluated because of their function and accumulation in G2/M phase transition [48, 49]. Downregulation of both cyclins confirmed cell cycle arrest results. This G2/M phase arrest caused by Cu-TA may be partially responsible for the growth inhibitory response in Panc1 cells. There was no cell cycle arrest observed in MIA PaCa-2 cells at either time point studied. Cu-TA could perhaps be predominantly affecting apoptotic pathways rather than cell cycle arrest in MIA PaCa-2 cells. Next Generation Sequencing analysis is currently under investigation to understand what other pathways are affected by Cu-TA treatment.

Because of our successful results in the *in vitro* assays, a pilot study using a mouse model for PaCa was done to confirm our findings in an *in vivo* model. The Cu-TA treatment dose dependently caused a decrease in tumor weights (25mg/kg: 48%; 50 mg/kg: 68%) (Fig. 7A). Apart from the reduction in tumor size and weight, there is also a visible decrease in tumor vasculature (Fig. 7A). This is significant since tumor vasculature is vital for tumor growth and survival [50]. Furthermore, there was no changes observed in the mice body weights (Fig. 7B), indicating that no obvious toxicity was occurring with Cu-TA treatment.

In summary, Cu-TA's IC_{50} dosage to inhibit cell proliferation was found to be half that of TA demonstrating higher efficacy and the growth inhibitory effect of Cu-TA was attributed to cell cycle arrest; demonstrated by an increase of Panc1 cells in G2/M phase. Activation of apoptosis was also shown (by increasing effector caspase activity, cleaved PARP

protein expression and apoptotic cell populations) and may contribute to the inhibition of proliferation. 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% (Fig. 2A&B). 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. However, other mechanisms could be involved and pathway analysis of Cu-TA treatment in PaCa cells is currently under investigation.

Conclusion

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. In this investigation, Cu-TA showed anti-cancer activity in PaCa cells and had an enhanced efficacy 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 growth inhibitor effect seen in our *in vitro* studies were reproducible in our mouse model for PaCa. Recent findings from our group [36] demonstrated the stable biological (anti-proliferative activity against PaCa cells) and structural stability as characterization by FTIR and UV-visible spectroscopy for up to 12 months. Additionally, Cu-TA did not cause any significant cytotoxicity in cardiomyocytes [36]. The cardiomyocyte data along with our mouse model results provides preliminary evidence that Cu-TA is non-toxic to non-malignant cells. 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. Therefore, allowing the patients to receive less chemotherapy and radiation for less side effects without compromising therapeutic efficacy. Further testing is required, but this provides preliminary evidence for the potential use of Cu-TA in PaCa treatment.

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Disclosure Statement

The authors have no conflicts of interest in this research.

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