COMBINATION THERAPY TO ENHANCE ANTI-CANCER ACTIVITY AGAINST NEUROBLASTOMA AND EWING SARCOMA CELLS

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DISSERTATION ABSTRACT

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Anti-neoplastic drugs used for the treatment of various cancers often cause severe toxicity. Chemotherapy induced side effects are highly detrimental in pediatric and adolescent and young adult (AYA) cancers. This project is aimed at evaluating the strategies to induce the therapeutic efficacy of anti-neoplastic agents in a pediatric and an AYA cancer models, neuroblastoma (NB) and Ewing Sarcoma (ES) respectively. NB is a most common type of pediatric cancer that arises from autonomic nervous system while ES is a very aggressive bone and soft tissue sarcoma diagnosed in AYA patients. Small molecules such as Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are under investigation for their anti-cancer activities. The transcription factor, Specificity protein 1 (Sp1) and an inhibitor of apoptosis protein, survivin are over-expressed in multiple cancers including NB and Sarcoma. Sp1 and survivin has been shown to be associated with poor prognosis and resistance to chemo-/radiation therapy in some cancer models. Tolfenamic acid (TA), a NSAID has been shown to induce the anti-proliferative activity of several cancer cells by downregulating Sp1 and survivin. Therefore, we hypothesized that targeting these candidates using TA will enhance the anti-proliferative activity of anti-neoplastic agents in NB and ES cells. After initial screening of anti-neoplastic agents using NB and ES cell lines, cis-retinoic acid (RA) was chosen for NB and vincristine (Vin) was selected for ES. Experiments were conducted using the NB and ES cell lines to assess the individual and

combination treatment of TA and anti-neoplastic agents (RA and Vin) and the results presented below in 3 specific aims.

Aim 1: Chemotherapy used for the treatment of NB often causes long-term side effects in pediatric patients. Moreover, resistance to chemotherapy and disease relapse is serious concerns in high-risk neuroblastoma (HRNB). RA is used as an anti-neoplastic agent to induce remission in HRNB. However, HRNB patients treated with RA suffers from severe toxicities and the relapse rate is very high. Therefore, it is important to develop alternative strategies for effective treatment of HRNB. In this aim we investigated the efficacy of TA, for enhancing the anti-proliferative effect of RA in NB cell lines. TA and RA combination treatment resulted in decreased Sp1 and survivin expression which was accompanied by decreased cell growth and increase in apoptosis. This study demonstrated that the TA-mediated inhibition of Sp1 in combination with RA provides a novel therapeutic strategy for the effective inhibition of HRNB cells.

Aim 2: In this aim, we demonstrated a strategy to target Sp1 and survivin using TA to inhibit ES cell growth. Our results revealed that TA inhibited cell viability, induced G0/G1 cell cycle arrest and increased apoptosis in ES cells. Mechanistically, we showed that that TA inhibited Sp1 and survivin protein and/or mRNA expression, disrupted Sp1 DNA-binding and inhibited ES cell proliferation. The results of this investigation suggest that targeting Sp1 and survivin could be an effective strategy for inhibiting ES cell growth.

Aim 3: Vincristine, a plant derived alkaloid, is an integral part of chemotherapeutic regimens used for the treatment of ES. Vin treatment is known to cause severe long-term side effects such as sensory and motor neuropathy. Therefore, there is a need to identify novel strategies to improve the efficacy and reduce toxicities associated with the use of Vin. We showed that TA enhanced anti-cancer activity of Vin in ES cells. Specifically, TA and Vin combination treatment decreased Sp1 and survivin protein expression which was accompanied by an increase in apoptotic markers and cell cycle (G2/M) arrest, thereby leading to ES cell growth inhibition. Results of this study demonstrated that TA could enhance the anti-proliferative activity of Vin and suggesting that TA+Vin combination could be more effective against ES cell growth.

Taken together, these data suggest that combination of TA and anti-neoplastic agent(s) treatment could be developed as a novel and safer therapeutic strategy for cancer treatment by targeting Sp1 and survivin.

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DISSERATATION

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CHAPTER I

DISSERTATION OVERVIEW

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs most commonly used as analgesics, antipyretics, and anti-inflammatory agents. Several studies have reported anti-cancer activity of NSAIDs against multiple cancer types (Juni, Reichenbach et al. 2005, Peterson, McDonagh et al. 2010). NSAIDs mediate their activity via inhibition of cyclooxygenase (COX) enzymes, Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) (Koki and Masferrer 2002, Peterson, McDonagh et al. 2010). Several research groups are investigating NSAIDs such as Tolfenamic Acid (TA), Ibuprofen, Sulindac, Naproxen, and Celecoxib as anti-cancer agents. TA has been shown to induce anti-proliferative activity in the cancer cells by downregulating specificity protein family transcription factors (Sp1, Sp3, and Sp4). Current observations from our laboratory and others indicate that TA, which acts via a COX-independent mechanism, inhibits growth and induces apoptosis in multiple cancers, such as medulloblastoma, neuroblastoma (NB), and leukemia by inhibiting expression of Sp1 and survivin (Eslin, Sankpal et al. 2013, Sutphin, Connelly et al. 2014).

We have previously shown that TA treatment inhibits the protein expression of Sp1 transcription factor resulting in attenuation of cancer cell growth and G0/G1 cell cycle arrest in a variety of cancer cells (Eslin, Sankpal et al. 2013, Sutphin, Connelly et al. 2014). Our laboratory is testing novel strategies for using TA as a sensitizing agent to enhance the anti-proliferative response of anti-neoplastic agents for the treatment of advanced and metastatic cancer types that are difficult to treat. The research work presented in this dissertation is an endeavor to investigate the effect of TA and anti-neoplastic agent(s), for the treatment of NB and Ewing sarcoma (ES).

In the first aim, we demonstrate that the combination of TA and cis-retinoic acid (RA) effectively inhibits cell growth and induces apoptosis in high-risk neuroblastoma cells. In the second aim, we investigate the molecular mechanism associated with the anti-proliferative activity of TA in ES cells. In the third aim, we show that TA enhances the anti-proliferative response of vincristine in ES cells. Following is a brief overview of NB and ES disease biology, and observations from previous and ongoing research in the development of new therapeutics pertaining to NB and ES.

Neuroblastoma:

NB is most common extra-cranial solid tumor that originates from the sympathoadrenal lineage of neural crest during embryonic development and is found in adrenal gland and peripheral nerve cells. This malignancy often arises in the adrenal glands but it is also occasionally found in the neck or spinal cord. The incidence of NB is higher in children younger than five years of age. Despite the advances in the tumor detection technologies, NB usually metastasize to other parts of the body by the time it is diagnosed (Matthay, Reynolds et al. 2009). Based upon the genetic make-up such as, MYCN amplification status, stage of diagnosis, and age, it is categorized as low risk, intermediate risk and high-risk NB (HRNB). Clinically, stage 1 and stage 2 categories represent as localized and comparatively well-differentiated tumor that can be cured by surgery. However, stage 3 and stage 4 category of tumors are often diagnosed when they are already disseminated, and hence are grouped as high-risk NB. HNRB are very difficult to treat and have poor prognosis due to their resistance to chemotherapy and very high relapse rate (Castleberry, Pritchard et al. 1997, Cohn, Pearson et al. 2009). Retinoids, also known as vitamin A, are usually used as a differentiating agent in the treatment regimens of

HRNB. To control the minimal residual diseases (MRD), HRNB cases are often managed with RA and combination of conventional chemotherapy drugs. Although treatment with RA improves survival of HRNB patients by 35%, a very high percentage of patients suffer from relapse and eventually succumb to death (Matthay, Reynolds et al. 2009). Therefore, there is an urgent need to develop a more effective treatment strategy to improve the long-term survival of NB patients. Previously we showed that TA treatment effectively induces anti-proliferative response in NB cells (Eslin, Sankpal et al. 2013). In the first aim, we investigated the effectiveness of TA and RA combination treatment against NB cells growth and proliferation.

Ewing Sarcoma:

ES is the second most common aggressive sarcoma of bone and/or soft tissue, that arises from mesoderm-derived mesenchymal stem cells. The incidence rate of ES peaks in adolescent and young adult population. It is characterized by the presence of EWS-FLI1 fusion protein. The chimeric EWS-FLI1 protein is formed by oncogenic fusion protein, between EWS RNA-binding protein 1 (EWS) gene on the 22q12 chromosome and E26 transformation specific transcription factor family proto-oncogene (ETS), FLI1, on chromosome 11q24. Importantly, 85% of ES tumors harbor EWS-FLI1 fusion protein created by t(11;22)(q24;q12) chromosomal translocation (Ghisoli, Barve et al. 2015). Several lines of evidence suggest that EWS-FLI1 is a highly potent transcription activator compared to wild type EWS or FLI1 transcription factors, and interacts with multiple transcription factors including Specificity protein 1 (Sp1), to contribute towards the aggressiveness of ES (May, Lessnick et al. 1993, Lessnick, Braun et al. 1995). Disruption of EWS-FLI1 activity by gene silencing techniques such as siRNA and small molecule inhibitor results in decreased tumor size and increased overall survival in ES xenograft

pre-clinical models. However, these approaches need more optimization to translate them to clinical therapy due to technical difficulties such as drug delivery system and drug stability issues (Erkizan, Kong et al. 2009, Jully and Rajkumar 2012, Kelleher and Thomas 2012).

Sp1 is a transcription factor that plays a key role in critical cellular processes such as cell cycle phase distribution, cell proliferation, and apoptosis. Previous studies have demonstrated that Sp1 regulates cell proliferation and apoptosis by modulating expression of survivin (a member of the inhibitor of apoptosis protein family), Vascular Endothelial Growth Factor (VEGF) and Cyclin D1 (Fuchs, Inwards et al. 2004, Giorgi, Boro et al. 2015). Moreover, high expression of Sp1 and survivin is associated with aggressiveness and poor prognosis in various cancer types including sarcomas (Fukuda and Pelus 2006). Although Sp1 is expressed in NB and ES and is viewed as a ‘'hallmark of cancer", strategies to target Sp1 are still lacking. Sp1 in co-operation with NF-kB (Nuclear Factor-kB), has been shown to regulate expression of Cyclin D1 protein and mRNA in various cell types (Klein and Assoian 2008, Marampon, Casimiro et al. 2008). Cyclin D1 plays a very important role in the regulation of G0/G1 to S phase cell cycle transition. In this regard, overexpression of Cyclin D1 allows the cancer cells to bypass normal cell cycle progression to enter into uncontrollable cell division. Overexpression of Cyclin D1 has been linked to the poor prognosis of various cancers including colon, breast, prostate, NB and ES (Fagone, Nicoletti et al. 2015, Kennedy, Vallurupalli et al. 2015, Magro, Brancato et al. 2015). As mentioned before, the second and third Aims of this dissertation focus on identifying the mechanisms of TA and studying the effectiveness of TA and Vincristine combination treatment for inhibiting ES cell proliferation. Previously, we have shown that TA treatment results in significant inhibition of Sp1 protein expression resulting in inhibition of cancer cell growth, and causes G0/G1 cell cycle arrest in a variety of cancer cells (Abdelrahim, Baker et al. 2006, Eslin,

Lee et al. 2013, Eslin, Sankpal et al. 2013). Therefore, we hypothesize that in ES cells, TA treatment will inhibit cell proliferation, induce cell cycle arrest and increase apoptosis by downregulating Sp1 and survivin.

Chemotherapeutic agents such as vincristine, etoposide, ifosfamide, cyclophosphamide, doxorubicin and dactinomycin are part of standard chemotherapy regimen for treating ES patients. The response to chemotherapy is often ineffective primarily due to drug resistance. Furthermore, the dose limiting toxicity associated with the chemotherapy is also a major concern. Also, there has been little or no improvement in the therapeutic intervention for ES over the last several years. Therefore, there is an urgent need to identify safer and more effective treatment options for ES treatment. In the third Aim, we investigated the effectiveness of combination of TA and Vincristine treatment against ES cell lines.

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CHAPTER: II

COMBINATION OF 13 CIS-RETINOIC ACID AND TOLFENAMIC ACID INDUCES APOPTOSIS AND EFFECTIVELY INHIBITS HIGH-RISK NEUROBLASTOMA CELL PROLIFERATION

ABSTRACT

Chemotherapeutic regimens used for the treatment of Neuroblastoma (NB) cause long-term side effects in pediatric patients. NB arises in immature sympathetic nerve cells and primarily affects infants and children. High rate of relapse in high-risk neuroblastoma (NB) necessitates the development of alternative strategies for effective treatment. This study investigated the efficacy of a small molecule, tolfenamic acid (TA), for enhancing the anti-proliferative effect of 13 cis-retinoic acid (RA) in NB cell lines. LA1-55n and SH-SY5Y cells were treated with TA (30 μM) or RA (20 μM) or both (optimized doses, derived from dose curves) for 48 h and tested the effect on cell viability, apoptosis and selected molecular markers (Sp1, survivin, AKT and ERK1/2). Cell viability and caspase activity were measured using the CellTiter-Glo and Caspase-Glo kits. The apoptotic cell population was determined by flow cytometry with Annexin-V staining. The expression of Sp1, survivin, AKT, ERK1/2 and c-PARP was evaluated by Western blots. The combination therapy of TA + RA resulted in significant inhibition of cell viability when compared to individual agents. The anti-proliferative effect is accompanied by a decrease in Sp1 and survivin expression and an increase in apoptotic markers, Annexin-V positive cells, caspase 3/7 activity and c-PARP levels. Notably, TA + RA combination also caused down regulation of phospho-AKT and phospho-ERK1/2 suggesting a distinct impact on survival and proliferation pathways via signaling cascades. This study

demonstrates that the TA mediated inhibition of Sp1 in combination with RA provides a novel therapeutic strategy for the effective treatment of NB in children.

Keywords: Sp1, Survivin, Tolfenamic acid, Retinoic acid, High-risk neuroblastoma, Combination therapy.

INTRODUCTION

Neuroblastoma (NB) is an extra-cranial solid tumor that originates from the sympatho-adrenal lineage of neural crest during embryologic development and is found in infants and children, most commonly under the age of 5 years. It is the most common pediatric solid tumor and accounts for approximately 15% of childhood cancer deaths (Hoehner, Gestblom et al. 1996). The complexity and aggressiveness of NB is determined by a number of factors such as disease stage, age at diagnosis, and cellular/genetic make-up of tumor and is categorized into low-, intermediate- and high-risk based on these factors (van Ginkel, Sareen et al. 2007, Cheung and Dyer 2013). The standard treatment for high-risk neuroblastoma (NB) includes surgery, intensive chemotherapy, irradiation, bone marrow transplant biologic therapy with isotretinoin (13-cis-retinoic acid or RA) and CH14.18 antibodies (Matthay, Reynolds et al. 2009). Despite recent improvements in therapeutic regimens, prognosis of high-risk neuroblastoma (NB) is still poor with approximately 50% mortality rate (Preis, Saya et al. 1988, Ebb DH 2001, Carosio, Zuccari et al. 2007, van Ginkel, Sareen et al. 2007, Maris 2010).

Retinoic Acid (RA) is currently used as adjuvant therapy. It is typically given during the maintenance phase of NB; however, the data from the Children's Cancer Group study (CCG-3891) indicated certain limitations of RA in improving event-free survival rate (Matthay, Reynolds et al. 2009). RA induces the differentiation of neuroblasts and skeletal myoblasts (Preis, Saya et al. 1988, Xun, Lee et al. 2012). RA inhibits cell growth and induces differentiation in NB cells including the cell lines established from refractory tumors. It has been suggested that RA effectively targets residual tumor cells that are resistant to chemo or radiation therapy. RA improves mitochondrial respiration and increases the metabolic rate in differentiated cells, while reducing the availability of nutrients to undifferentiated cells and suppresses cell

growth (Xun, Lee et al. 2012). Even though RA has clinical advantages in NB, it also has several limitations. Inter-and intra-patient variations in the RA plasma concentration and pharmacokinetics have been reported (Veal, Cole et al. 2007, Veal, Errington et al. 2013). In addition, RA can cause acute and long term toxicity in various biological functions (van Ginkel, Sareen et al. 2007). In order to improve cellular response to RA in NB treatment, drug combination therapies leading to reduced toxicity and increased event free survival are currently being explored.

Specificity protein1 (Sp1) is the first mammalian transcription factor to be cloned (Black, Black et al. 2001). Over the past decade, molecular studies conducted on Sp1 demonstrated the crucial role of Sp1 in the regulation of cell growth, differentiation and apoptosis. Elevated levels of Sp1 have been implicated in the development of various cancers and several lines of evidence suggest that the correlation between Sp1 expression and poor prognosis in cancer patients (Chang and Chen 2005, Abdelrahim, Baker et al. 2006, Lu and Archer 2009, Basha, Baker et al. 2011). In addition, previous studies have shown that Sp1 transcription factor regulates the expression of survivin, a key mediator of apoptosis (Mita, Mita et al. 2008, Doolittle Helen 2010). Recently we have shown that Sp1 is expressed in NB clinical specimens and targeting Sp1 with the small molecule/non-steroidal anti-inflammatory drug (NSAID) Tolfenamic Acid (TA) inhibits NB cell proliferation.

In this study, we have investigated the individual and combined effect of TA and RA for inhibiting NB cell growth. NB cells, LA1-55n and SHSY-5Y, were treated with optimized concentrations of TA, RA, TA+RA and cell viability was measured at 24 and 48 h post-treatment. In order to understand the effect on apoptosis, critical markers (caspase 3/7 activity,

expression of cleaved PARP and percentage of apoptotic cell population) were monitored. The expression of Sp1, survivin, AKT and ERK1/2 was also determined.

MATERIAL AND METHODS

Cell Lines

LA1-55n (*MYCN amplified*) and SH-SY5Y (*MYCN non-amplified*) cell lines are derived from bone metastases of childhood cancer with high-risk neuroblastoma. SH-SY5Y is a mixed culture, containing both adherent and suspended cells. Cells were grown in RPMI 1640 media (ATCC) with fetal bovine serum and supplemented with 100U/ml penicillin and 100 U/ml streptomycin. All cultures were maintained at 37°C and 5% CO_2 as previously described (Saulnier Sholler, Brard et al. 2009).

Chemicals and Reagents

Antibodies for Sp1 and survivin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). c-PARP, ERK1/2, pERK1/2, AKT and pAKT antibodies were acquired from Cell Signaling Technology (Danvers, MA) while β-actin was obtained from Sigma Chemical Co. (St. Louis, MO). Annexin-V/7-AAD kit for apoptosis was purchased from BD Biosciences (San Jose, CA). Tolfenamic acid (TA), dimethyl sulfoxide (DMSO), 13-cis-retinoic acid (RA) and protease inhibitor were purchased from Sigma. Dulbecco's phosphate-buffered saline (PBS) was purchased from Hyclone Laboratories (Logan, Utah), and cell lysis buffer was obtained from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) protein assay kit and Supersignal West Dura were purchased from Pierce (Rockford, IL) and Caspase-Glo 3/7 CellTiter-Glo kits were obtained from Promega (Madison, WI).

Cell Viability

The NB cells were grown in RPMI1640 media and treated with DMSO (control) or TA or RA or TA and RA and cell viability was measured using Cell Titer-Glo kit (Promega, Madison, WI). Briefly, 4000 cells /well were plated in 96-well white walled clear bottom plates (Lonza, Basel, Switzerland) and incubated for 48 h, unless otherwise noted, at 37°C. LA1-55n and SH-SY5Y cells were treated with increasing concentration of TA (10, 20, 30, 40, 50 and 60 µM) or RA (10, 20, 30, 40 and 50 µM) for 24 h and 48 h. At the end of the incubation period, 100 µl of assay reagent was added, mixed, and the plate was incubated in the dark for 20 min. Luminescence values were obtained from each well using SYNERGY HT microplate reader. All treatments were performed in triplicate and the data was normalized for control (DMSO-treated) cells and plotted as percent cell viability versus drug concentration.

Combination Index Analysis:

Logarithmically growing cells (4000 cells/well) were plated in 96-well white walled clear bottom plates (Lonza, Basel, Switzerland) and grown at 37°C in a cell culture incubator. Cells were treated with fixed ratio TA/RA (2:3) for 48 h and cell viability was measured using Cell Titer-Glo kit. The dose-effect relationship analysis for TA and RA was calculated to determine the combination index (CI) by CalcuSync software (BIOSOFT, Cambridge, UK), where CI values <1, =1 and >1 represents synergistic effects, additive effects, antagonistic effects respectively.

Cell Apoptosis using Flow Cytometry

Apoptotic cells were measured using Annexin-V/7-AAD apoptosis detection kit (BD Biosciences). Briefly, cells were harvested after treatment with vehicle (control) or individual drug (TA or RA), or combination of investigational agents for 48 h. Cells were incubated with annexin-V antibody and 7-AAD for 15 min in 1X binding buffer and cells in various populations (e.g., pre-apoptotic, apoptotic and dead) were analyzed using BD LSR II flow cytometer. Data was analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR).

Caspase activation assay

LA1-55n and SH-SY5Y cells were treated with TA or RA or TA+RA and the activity of caspase 3/7 was evaluated using Caspase-Glo 3/7 kit (Promega, Madison, WI), according to manufacturer instructions. Briefly, 4,000 cells/well were plated in a 96-well white walled clear bottom plate (Lonza, Basel, Switzerland) in two sets (cell viability and caspases 3/7). After 24 h, cells were treated with DMSO (control) or TA or RA, or TA+RA (with chosen drug concentrations). Following 48 h post-treatment, the assay (Cell Titer-Glo or Caspase-Glo 3/7) reagent (100 µl/well) was added, mixed and the plates were incubated in the dark for 60 min. Luminescence was measured using SYNERGY HT microplate reader. The activity of caspases was normalized with cell viability. All the treatments were performed in triplicates, and the data were presented as mean ± SD.

Western Immunoblotting Analysis

NB cells were cultured in 100 mm dishes and treated with DMSO (control) or TA or RA or TA + RA. Whole cell lysates were prepared and the expression of proteins of interest was determined through Western blot analysis. Briefly, cells were harvested at 48 h post-treatment,

washed twice with ice-cold PBS, and re-suspended in cell lysis buffer containing protease inhibitor. Samples were incubated at 4°C for 30 min, followed by centrifugation at 12,000 rpm for 15 min at 4°C. Protein estimation was performed using BCA protein assay kit. Protein extracts (25-30 µg protein) were boiled with loading buffer containing 2-Mercaptoethanol, separated using 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The blots were blocked with blocking buffer [5 % (w/v) nonfat dry milk in 10 mmol/L Tris (pH 7.5), 10 mmol/L sodium chloride, and 0.1% Tween 20 (TBST) for 30-60 min at room temperature. Blots were incubated with the primary antibody for overnight at 4°C. The antibody labeled blots were washed three times with TBST for 15 min and incubated with 1:5000 dilution of horse-radish peroxidase (HRP) conjugated secondary antibody for 2 h. Bands were visualized using Supersignal West Dura using UVP, LLC Bio-Imager.

Statistical Analysis

All experiments were performed with triplicates. All quantitative data was represented as mean±SD. Statistical analysis was performed using one-way ANOVA and statistical significance was calculated. Differences were considered as statistically significant at p value < 0.05. All data analysis was performed using GraphPad Prism V6.0 (La Jolla, California, USA)

RESULTS

Tolfenamic Acid and Retinoic Acid attenuated neuroblastoma cell growth

The anti-proliferative activity of TA and RA was investigated using two NB cell lines, LA1-55n and SH-SY5Y. To establish the working concentrations for RA and TA, we first

treated NB cell lines with increasing concentrations of TA (10-60 μM) or RA (10-50 μM) and cell viability was assayed at 24 and 48 h post-treatment. Both TA and RA resulted in a dose and time dependent inhibition of cell viability in LA1-55n and SH-SY5Y cell lines (Figure 2.1). The IC_{50} values for TA for these cell lines ranged between 30-40 μM whereas that of RA ranged between 15-25 μM at 48 h post-treatment. Our results indicate that NB cells are sensitive to clinical/therapeutic concentrations of TA and RA. After analyzing the data, 30 μM of TA and 20 μM of RA was chosen for the combination treatment. At these chosen concentrations, SH-SY5Y cell line showed a synergistic response in combination treatment (CI value at ED_{75}: 0.865; CI value at ED_{90}: 0.641) (Table No. 2.1).

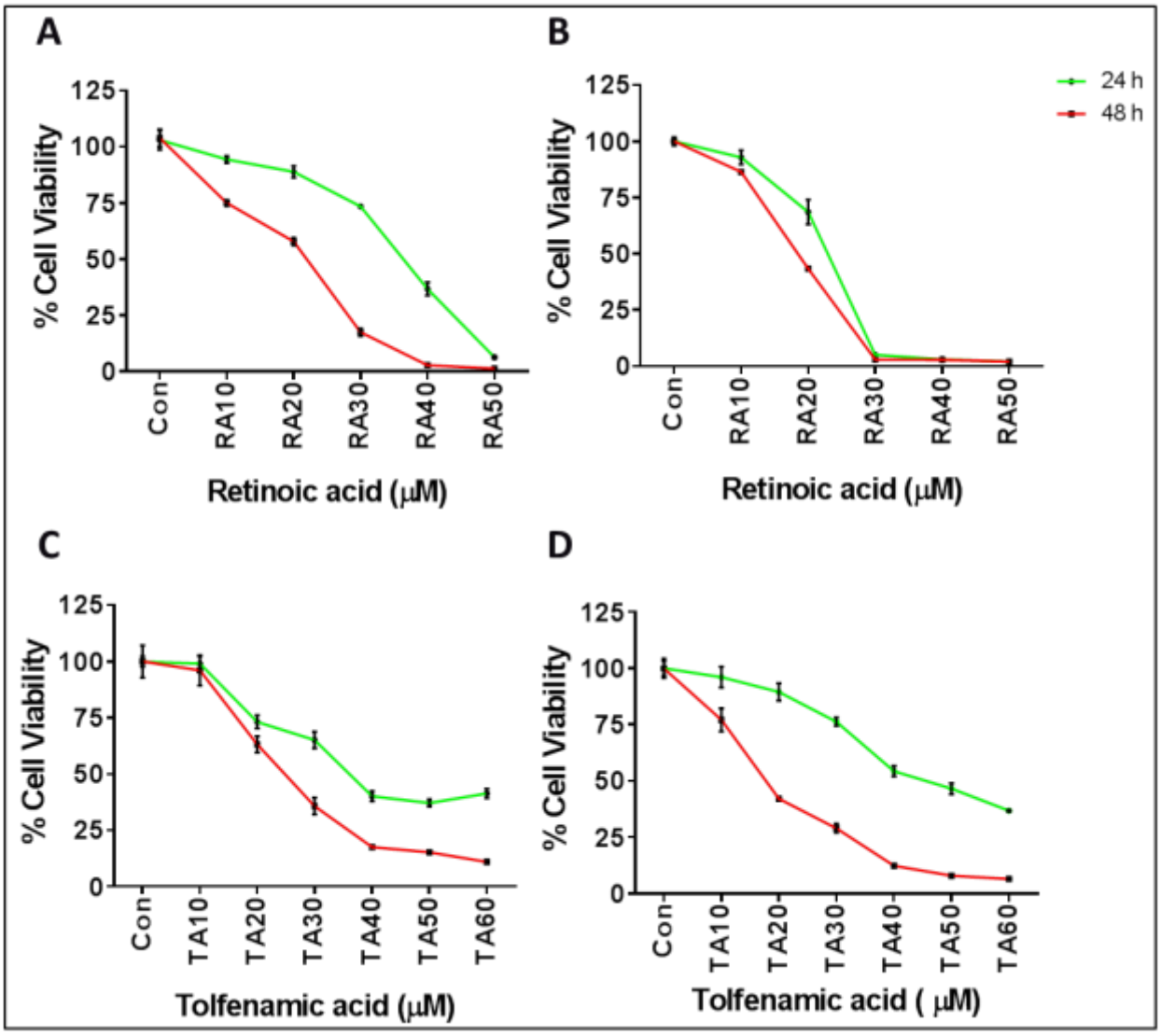

Figure 2.1: TA and RA inhibit cell viability of NB cells. Human neuroblastoma cells, LA1-55n (A and C), SH-SY5Y and (B and D) were treated with DMSO (Control) or increasing concentrations (10, 20, 30, 40 and 50 μM) of RA or TA and the cell viability assay was performed using CellTiter-Glo kit (Promega) at 24 h and 48 h post-treatment. Data shown indicate the percent cell death over control (DMSO). Bars represent the mean ± SD of three independent determinations.

Cell Line	CI at ED_{50}	CI at ED_{75}	CI at ED_{90}
LA1-55n	1.087	1.025	1.053
SH-SY5Y	1.29	0.865	0.641

Table No 2.1: LA1-55n and SH-SY5Y cell lines were treated with TA and RA alone or with combinations at various doses. Combination Index was calculated using CompuSync Software (BIOSOFT, Cambridge, UK). CI values <1, =1 and >1 represents synergism, additive and antagonism respectively.

Combined treatment of Tolfenamic acid and Retinoic acid inhibits the expression of Sp1 and survivin

The anti-proliferative effect of optimized doses of TA (30 μM) and of RA (20 μM) was tested in LA1-55n and SH-SY5Y cells. TA + RA combination treatment significantly inhibited cell growth as compared to individual agents (Figure 2.2 A & B). The effect of TA and RA alone or TA+RA combination treatment on the expression of Sp1 and survivin was determined by Western blot analysis. The results revealed that the cells treated with a combination of TA + RA had higher inhibition of Sp1 and survivin expression in both LA1-55n and SH-SY5Y (Figure 2.2) cells than either agent alone.

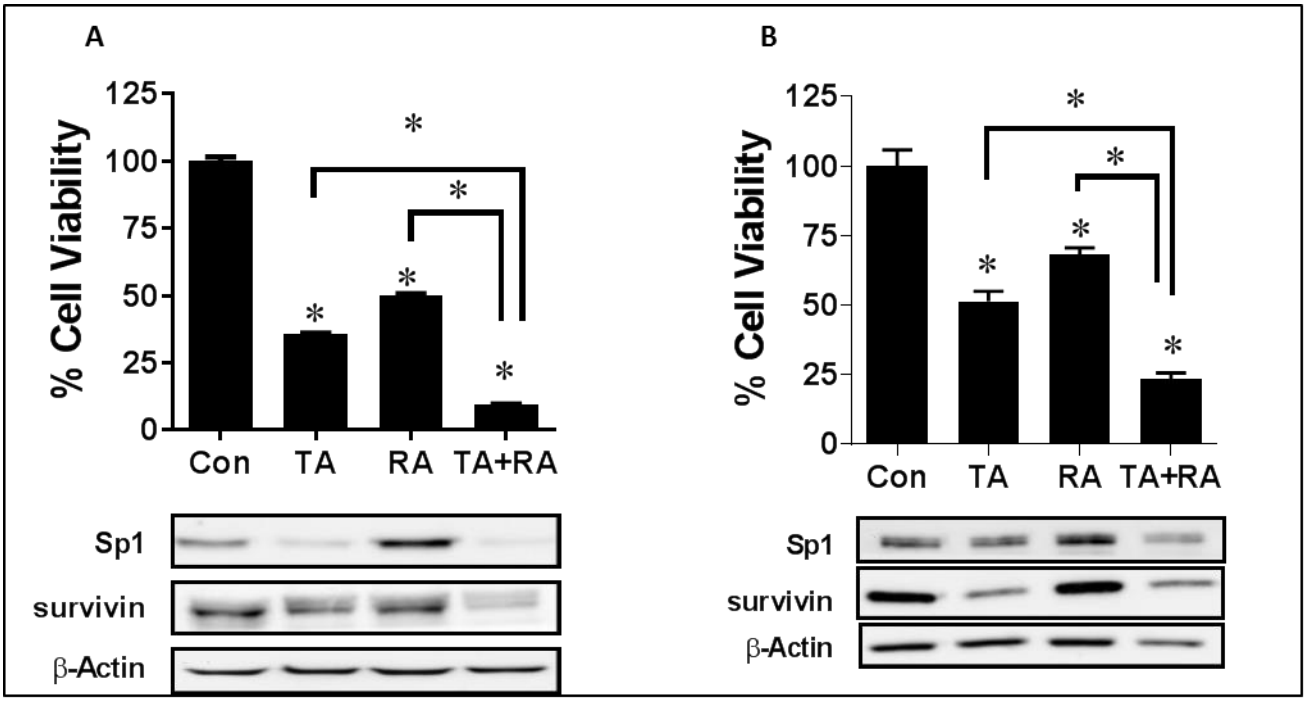

Figure 2: Co-treatment with TA and RA inhibits the expression of Sp1 and survivin. LA1-55n (A), SH-SY5Y (B) cells were treated with DMSO, TA (30 μM), RA (20 μM) or combination of TA + RA. Cell viability was assessed using CellTiter-Glo kit (Promega) at 48 h post-treatment. Whole cell lysates were prepared at 48 h post-treatment and the expression of Sp1 and Survivin was evaluated by Western blot analysis. β-actin was used as loading control. The data were obtained from at least three independent experiments and representative gels are shown in the figure. All data represent the mean ± SD. Statistical analysis performed by one-way ANOVA using GraphPad PrismV6.0 *(* represents p value < 0.05*).

CON: Control (DMSO treated); TA: Tolfenamic acid 30μM; RA: Retinoic acid 20μM. TA + RA: Tolfenamic acid 30 μM+ Retinoic acid 20μM.

Tolfenamic acid and Retinoic acid combination treatment induces cell apoptosis

NB cells, LA1-55n and SH-SY5Y were treated with a single drug or with the combination of TA + RA. The effect of TA + RA combination treatment on cell apoptosis was determined by flow cytometry using Annexin-V labeling assay. As determined by flow cytometry (Figure 2.3), the combination of TA + RA resulted in 48% and 33% apoptotic cells respectively in LA1-55n (Figure 2.3 A) and SH-SY5Y (Figure 2.3B) cells.

Recent studies have indicated that TA induced apoptosis is accompanied by caspase activation. To demonstrate that combination treatment involves higher apoptosis as compared to TA or RA alone, we have determined the expression of cleaved-PARP (a substrate cleaved by caspase during apoptosis) via immunoblot analysis and measured the activity of caspase3/7 in LA1-55n and SH-SY5Y cells. As shown in Figure 4, combination treatment resulted in a significant activation of caspse3/7 and upregulated the cleavage of PARP as compared to TA or RA alone in both LA1-55n (Figure 2.4A) and SH-SY5Y (Figure 2.4B) cell lines. TA+RA combination treatment caused several fold increase in caspase3/7 activity in both LA1-55n (12 fold, Figure 2.4A) and SH-SY5Y (9 fold, Figure 2.4B) cells, at 48 h post-treatment. In fact, the changes observed in the activation of caspase-3/7 are consistent with an increase in c-PARP expression (Figure 2.4) and an increase in apoptotic cell population (Figure 2.3).

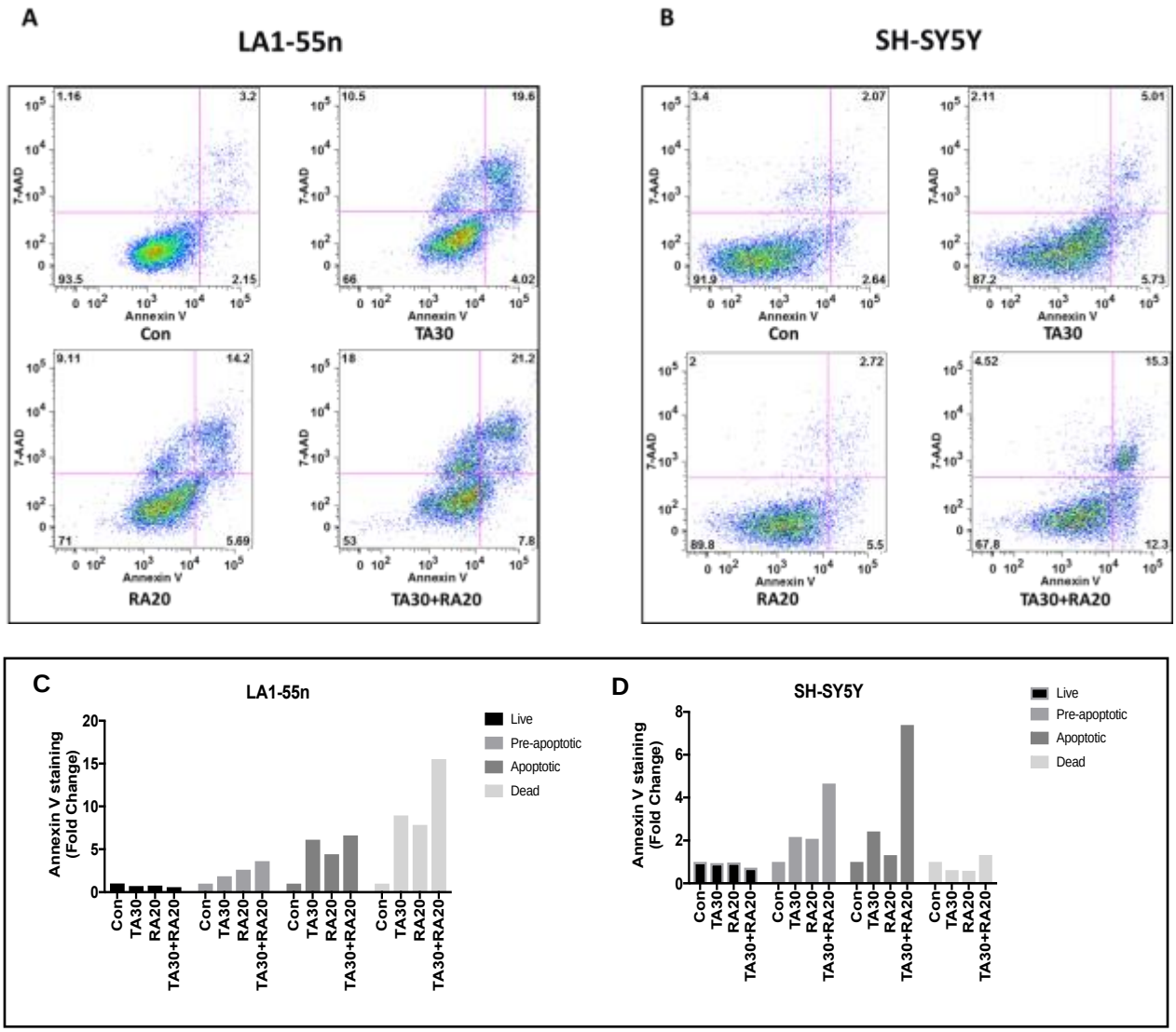

Figure 2.3: Co-treatment with Tolfenamic acid and Retinoic acid induces apoptosis. LA1-55n (A, C), SH-SY5Y (B, D) cells were treated with DMSO, TA (30 µM), RA (20 µM) or TA+RA combination and apoptosis was analyzed using Annexin V-PE/7-AAD kit 48 h post-treatment on BD LSR II (BD Bioscience).

CON: Control (DMSO treated); TA: Tolfenamic acid 30 µM; RA: Retinoic acid 20 µM. TA+RA: Tolfenamic acid 30 µM +Retinoic acid 20 µM

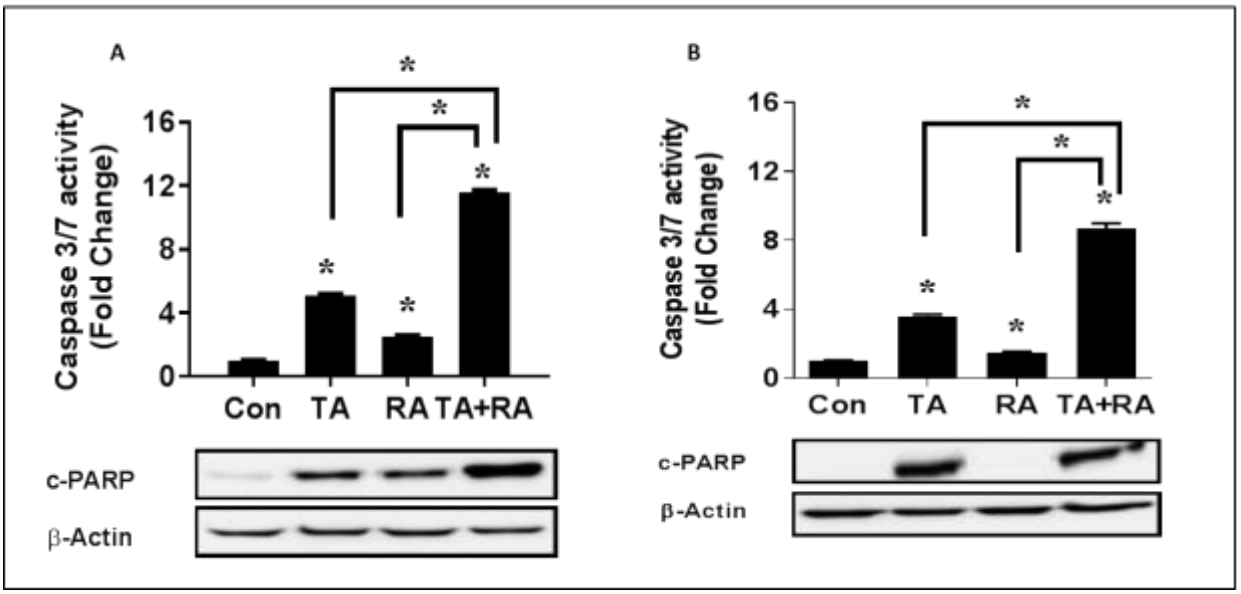

Figure 2.4: Tolfenamic acid and Retinoic acid combination treatment significantly up-regulated caspase-3/7 activity and c-PARP expression. LA1-55n (A), SH-SY5Y (B) cells were treated with DMSO, TA (30 μM), RA (20 μM) or TA+RA combination. Caspase 3/7 activity was measured using caspase 3/7 Glo kit at 48 h post-treatment. Whole Cell lysates were prepared and the expression of c-PARP was evaluated by Western blot analysis at 48 h post-treatment. The data were obtained from at least three independent determinations and representative gels are shown in the figure. Bars represent the mean ± SD of three independent determinations. Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0 *(*p<0.05*).

CON: Control (DMSO treated); TA: Tolfenamic acid 30 μM; RA: Retinoic acid 20 μM. TA+RA: Tolfenamic acid 30 μM +Retinoic acid 20 μM.

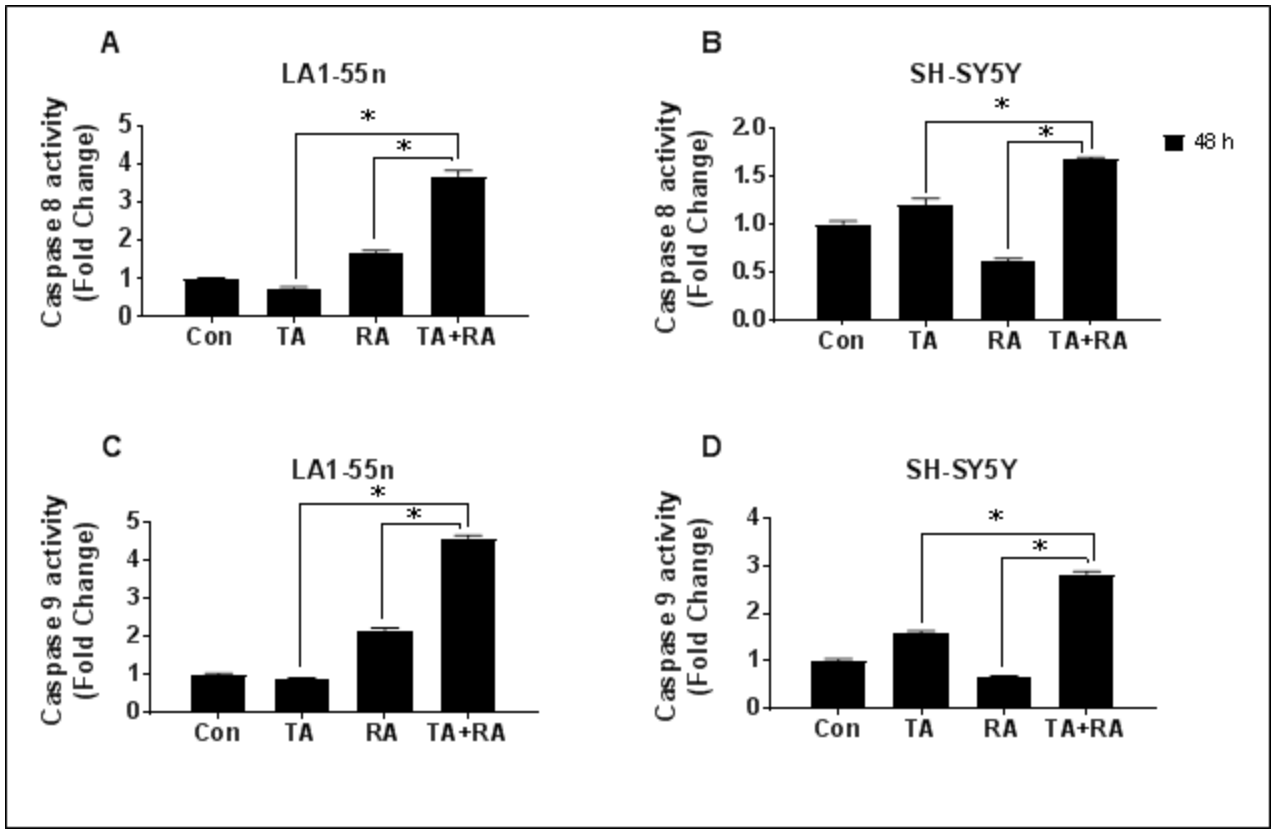

Figure 2.5: Tolfenamic acid and Retinoic acid combination treatment significantly up-regulated caspase-8 and caspase-9. (A, B) La1-55n and (C, D) SH-SY5Y cells were treated with DMSO, TA (30 µM), RA (20 µM) or TA+RA combination. Caspase-8 and caspase-9 activity was measured using caspase Glo-8 and caspase Glo-9 kit at 48 h post-treatment. Bars represent the mean ± SD of three independent determinations. Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0 *(*p<0.05*).

CON: Control (DMSO treated); TA: Tolfenamic acid 30 µM; RA: Retinoic acid 20 µM. TA+RA: Tolfenamic acid 30 µM +Retinoic acid 20 µM.

Combination treatment with Tolfenamic acid and Retinoic acid inhibits AKT and ERK1/2 signaling pathway

AKT and ERK1/2 signaling pathways play important role in cell growth, differentiation and apoptosis. In order to study the effects of the TA+RA combination treatment induced changes in AKT and ERK1/2 signaling pathways, we analyzed their phosphorylated status (pAKT and pERK1/2) along with total protein in NB cells. Consistent with the published results (Lopez-Carballo, Moreno et al. 2002), RA treatment induced phosphorylation of AKT (Figure 2.6). TA + RA combination treatment reduced pAKT in SH-SY5Y cells whereas pERK1/2 was decreased in LA1-55n cells. The downregulation of total AKT and ERK1/2 could be due caspase mediated degradation of the signaling molecules. These observations, then, show that inhibition of AKT and ERK1/2 signaling pathways may be, directly or indirectly, regulating TA+RA combination treatment induced apoptotic response in NB cells.

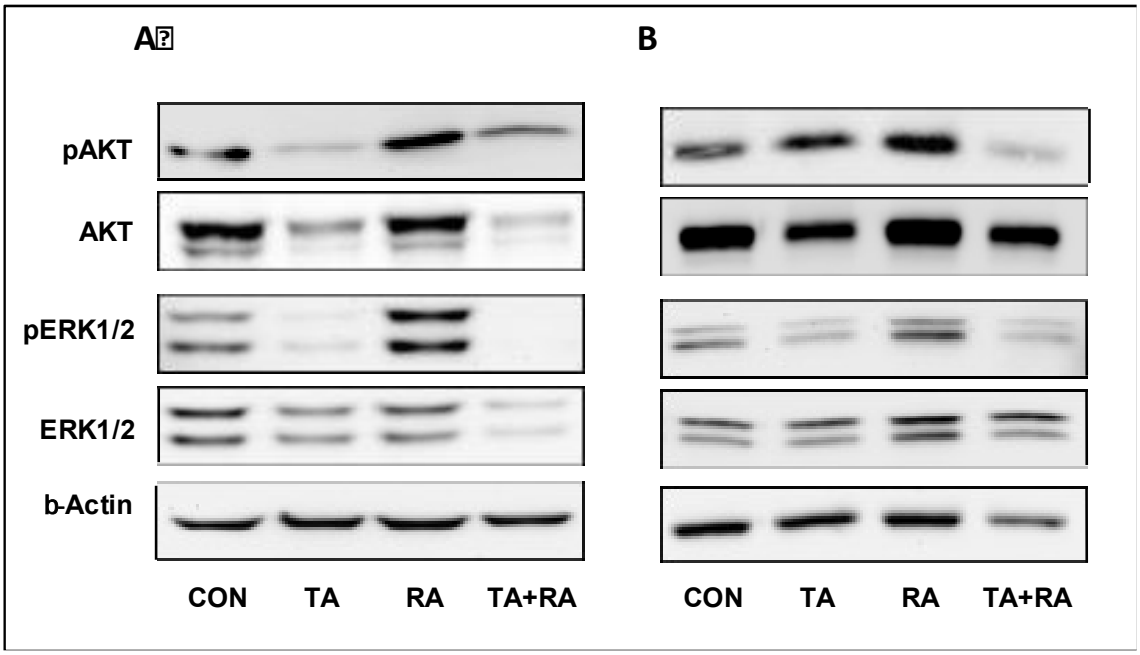

Figure 2.6: Tolfenamic acid and Retinoic acid co-treatment inhibits expression of AKT and ERK. LA1-55n (A), SH-SY5Y (B) cells were treated with DMSO, TA (30 µM), RA (20 µM) or TA+RA combination for 48 h. Whole cell lysates were prepared and the expression of AKT,

pAKT, ERK1/2 and pERK1/2 was evaluated by Western blot analysis. The data were obtained from three different determinations and representative gels are shown in the figure. β-actin was used as loading control.

CON: Control (DMSO treated); TA: Tolfenamic acid 30 μM; RA: Retinoic acid 20 μM. TA+RA: Tolfenamic acid 30 μM +Retinoic acid 20 μM.

DISCUSSION

Even with intensive treatment, NB may relapse in more than 50% of patients and majority of these patients also develop resistance to chemotherapy (Reynolds, Wang et al. 2000). RA has been used as part of standard treatment options for NB patients since the adjuvant therapy with RA has been shown to induce growth arrest and differentiation of NB cell lines (Sidell, Altman et al. 1983, Thiele, Reynolds et al. 1985). Despite the widespread interest for using RA in NB treatment, there are certain concerns in its clinical use. Toxicity, specificity and drug potency often limits the use of RA as a single anti-neoplastic agent (van Ginkel, Sareen et al. 2007). Various lines of evidence suggest that RA in combination with other therapies might pro-vide a better alternative to current NB chemotherapy treatments (Clark, Daga et al. 2013, Nolting, Giubellino et al. 2014). In this investigation we have attempted to test a strategy using a combination therapy involving a small molecule inhibitor of Sp1 along with RA.

Sp transcription factors regulate multiple genes involved in cellular processes including cell growth, apoptosis, invasion and metastasis. These factors are over-expressed in various cancers and have also been linked with poor prognosis and aggressive disease; Interestingly various other research groups have identified an association between Sp expression and aggressiveness of NB (Seeger, Brodeur et al. 1985, Look, Hayes et al. 1991, Nakagawara, Arima-Nakagawara et al. 1993, Eggert, Grotzer et al. 2002, Tang, Zhao et al. 2006). Therefore, targeting Sp transcription factors may serve as an ideal approach for inducing therapeutic efficacy (Basha, Baker et al. 2011). TA has been identified as an effective small molecule to target Sp transcription factors in preclinical testing (Abdelrahim, Baker et al. 2006, Abdelrahim, Safe et al. 2006, Papineni, Chintharlapalli et al. 2009, Basha, Ingersoll et al. 2011, Colon, Basha et al. 2011). Our lab has previously shown that TA causes cell growth inhibition and induces

apoptosis in NB cell lines potentially through inhibition of Sp1 and survivin expression (Eslin, Sankpal et al. 2013). In the present study, we analyzed the effect of RA combined with TA to target Sp1 and its downstream effectors in NB cell lines, LA1-55n and SH-SY5Y. Notably, our results demonstrate that the combined treatment of TA and RA resulted in a marked reduction of cell viability following a clear trend to show a dose and time dependent response (Figure 2.1).

We have previously shown that there are detectable levels of Sp1 expression in clinical specimens (biopsies from NB patient specimens) (Eslin, Sankpal et al. 2013). Consistent with our previously reported observations, TA treatment decreased Sp1 expression and cell viability in tested cell lines at 48 h post-treatment (Figure 2.2); however, TA and RA combination treatment resulted in a significantly greater decrease in Sp1 protein expression as compared to single drug alone at 48 h post-treatment (Figure 2.2). Although RA treatment resulted in 35 to 50% inhibition of cell viability, it had no effect on Sp1 expression. The analysis of combination index revealed that TA and RA combination treatment synergistically inhibits cell growth (Table 2.1) in SH-SY5Y cells. Survivin, a critical inhibitor of apoptosis inducing protein, has been reported to be upregulated in various cancers and associated with aggressive disease and poor prognosis. It also induces resistance to chemotherapy and radiation therapy. We observed a significant decrease in the expression of survivin following TA treatment. Survivin expression was further attenuated with combination treatment of TA and RA (Figure 2.2). Since, Sp1 mediates the expression of survivin (Li, Xie et al. 2006, Xu, Zhang et al. 2007), these results indicate that TA might sensitize these cells to RA via down-regulating Sp1.

The effect of combination treatment on apoptosis was evaluated by measuring the percentage of apoptotic (Annexin-V positive) cell population, activity of effector caspases and the expression of c-PARP at 48 h post-treatment. Consistent with our previous observations,

flow cytometric analysis showed a significant increase in Annexin-V stained cells following TA+RA combination treatment as compared to single agent. This combination treatment resulted in 5- and 3-fold increase in apoptotic cells respectively in LA1-55n (Figure 2.3A) and SH-SY5Y (Figure 2.3B) cells as compared to control cells. Caspases play central role in regulation of apoptosis. Both intrinsic and extrinsic apoptotic signals can activate initiator caspases. The activation of initiator caspase triggers the release of mitochondrial cytochrome C followed by activation of effector caspases 3 and 7. Consistent with our Annexin-V apoptosis results, we observed a significant increase in caspase 3/7 activity in the cells treated with TA+RA when compared to TA or RA alone (Figure 2.4). The expression of pro-apoptotic marker, c-PARP, showed a differential response in LA1-55n (Figure 2.4A) and SH-SY5Y (Figure 2.4B) cells. In this experiment, we observed that RA alone resulted in upregulation of c-PARP in LA1-55n, but we did not see a similar trend in SH-SY5Y cell line. The mechanism of action of RA involves downregulation of MYCN expression in MYCN amplified cell lines and is also shown to cause G0/G1 cell cycle arrest by transcriptional upregulation of p21/WAF1/CIP1 expression (Tanaka, Suh et al. 2007, Aktas, Altun et al. 2010). One possible explanation for differential induction of c-PARP could be that the inhibition mechanism of RA on cell growth is cell type specific.

Survivin, a key member of inhibitors of apoptosis (IAP) protein, is often found to be up-regulated in various human cancers including NB (Adida, Berrebi et al. 1998, Islam, Kageyama et al. 2000, Obexer, Hagenbuchner et al. 2009) and is known to inhibit effector caspases (caspase 3/7) (Grossman, Kim et al. 2001, Shankar, Mani et al. 2001, Zhang, Gillespie et al. 2004, Obexer, Hagenbuchner et al. 2009). TA and RA alone and TA+RA combination treatment induced down regulation of survivin may explain the marked upregulation in effector caspases

(caspase 3/7) as well as caspase-8 and caspase-9 (Figure 2.5 and Figure 2.5) and the resulting synergistic apoptotic response in NB cell lines.

AKT and ERK signaling pathways are known to play a central role in various cellular processes such cell survival, differentiation, apoptosis etc. and their deregulation may lead to the development and/or progression of cancer. AKT acts as a survival factor by exerting its anti-apoptotic effect on the release of cytochrome C from mitochondria while ERK signaling is well established as a regulator of cell proliferation (Zhang and Liu 2002, Whang, Yuan et al. 2004). Having observed a significant upregulation of effector caspases and inhibition of cell viability upon TA+RA combination treatment, we further evaluated the effect of TA and RA alone or TA+RA combination treatment on AKT and ERK signaling pathways. RA has been reported to induce AKT and ERK phosphorylation in NB cell lines (Qiao, Paul et al. 2012). Consistent with the previously reported data, we observed that RA upregulated phosphorylation of AKT and ERK in tested cell lines (Figure 2.6). Interestingly, TA + RA combination treatment causes dramatic downregulation of both phosphorylated and total AKT expression in both NB cells, however we did not observe such trend in ERK1/2 (Figure 2.6). AKT has been reported to be inhibited via proteasome dependent mechanism. On other hand, caspase dependent cleavage has also been shown to cause AKT inhibition (Bachelder, Wendt et al. 2001, Basso, Solit et al. 2002, Solit, Basso et al. 2003, Riesterer, Zingg et al. 2004). Alternatively, reactive oxygen species are reported cause caspase independent downregulation of AKT pathway by activating proteases (Martin, Salinas et al. 2002). Our results indicate that TA treatment alone and TA+RA combination treatment caused significant increase in caspase 3/7 activity (Figure 2.4). TA has been shown to cause activation of proteasome dependent degradation of Sp1 in pancreatic cancer cell line (Abdelrahim, Baker et al. 2006). Collectively, our results and previously published

literature suggest that observed inhibition of AKT signaling pathway could be attributed to the caspase 3/7 activation and participation of proteasome dependent degradation. Therefore, the distinct mechanisms of action of TA and RA may be contributing towards their ability to effectively inhibit cell growth and induce apoptosis when used as a combination treatment *in-vitro*. However, the specific molecular mechanism underlying the synergistic effect of TA+RA combination treatment in inhibiting NB cell proliferation remains to be determined.

Taken together, the current investigation using a combination of a small molecule (NSAID) TA and anti-neoplastic agent (RA) showed an effective anti-proliferative response on NB cells. These *in-vitro* experiments provide preliminary evidence for the efficacy of an effective alternative therapeutic option for the treatment of NB in children. Further studies to validate these results via *in vivo* assays are important and our future work will focus on this aspect. Unlike other NSAIDs, TA is not expected to cause additional toxicity or gastro-ulcerogenicity (Eskerod 1994) and could serve as a promising investigational agent for cancer treatment. Using TA along with RA in adjuvant therapy to target a key transcription factor associated with poor prognosis provides a novel approach to delay progression and prevent the relapse of NB. Our laboratory results provide a strong rationale for the use of TA + RA in combination treatments and in the development of effective combination therapies for treatment of NB and potentially for other such cancers.

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CHAPTER III

TARGETING SPECIFICITY PROTEIN 1 TRANSCRIPTION FACTOR AND SURVIVIN USING TOLFENAMIC ACID FOR INHIBITING EWING SARCOMA CELL GROWTH

ABSTRACT

Specificity protein1 (Sp1) transcription factor and its downstream target survivin (inhibitor of apoptosis protein), play major roles in the pathogenesis of various cancers. Ewing Sarcoma (ES) is a second most common soft tissue/bone tumor in adolescent and young adults. Overexpression of survivin is also linked to the aggressiveness and poor prognosis of ES. Small molecule-Tolfenamic acid (TA) inhibits Sp1 and survivin in cancer cells. In this investigation, we demonstrate a strategy to target Sp1 and survivin using TA and Mithramycin A (Mit), positive control, to inhibit ES cell growth. Cell viability was assessed with CellTiter-Glo assay. Flow cytometry was used to analyze cell cycle phase distribution with propidium iodide staining and apoptosis by Annexin V staining assay. Apoptosis markers: Caspase 3/7 activity, cleaved PARP protein (c-PARP), and cell cycle marker (Cyclin D1 protein) were determined. The mRNA and protein expression of Sp1 and survivin was determined by quantitative polymerase chain reaction (qPCR) and Western blot analysis respectively. Sp1 DNA-binding was evaluated by gel shift assay. Results revealed that knock down of Sp1 using small interfering RNA (siRNA) resulted in significant inhibition of CHLA-9 and TC-32 cell growth as assessed by CellTiter-Glo assay kit. TA treatment caused dose/time-dependent inhibition of cell viability and this inhibition was correlated with a decrease in Sp1 and survivin protein levels in ES cells. TA treatment increased Annexin-V positive cell population, caspase 3/7 activity, and upregulated c-PARP protein expression. Cyclin D1 is crucial for moving the cells from G0/G1 cell cycle

transition. Results indicated that TA induced G0/G1 cell cycle arrest and inhibited Cyclin D1. Quantitative PCR results showed that Mit treatment decreased mRNA expression of both survivin and Sp1, whereas TA diminished only survivin but not Sp1. Proteasome inhibitor restored TA-induced inhibition of Sp1 protein expression suggesting that TA might be causing proteasome-dependent degradation. Gel shift assay using ES cell nuclear extract and biotinylated Sp1 consensus sequence revealed that both TA and Mit decreased DNA-binding activity of Sp1. These results demonstrate that TA reduces expression of Sp1 and survivin, disrupt Sp1 DNA-binding and inhibit ES cell proliferation. This investigation suggests that targeting Sp1 and survivin could be an effective strategy for inhibiting ES cell growth.

Keywords: Ewing sarcoma, Sp1, Survivin, Tolfenamic acid, Apoptosis, Cell cycle, Targeted therapy.

INTRODUCTION

Sarcomas are the cancers arising from tissues of mesenchymal origin. According to American Cancer Society's estimation, approximately 12,000 cases and 5,000 deaths occur annually in the United States due to these malignancies. Currently, more than 50 distinct subtypes of sarcomas are identified based on morphological, immunohistochemical and molecular characteristics (S. Ushigome 2002, Jo and Fletcher 2014, Jo and Doyle 2016). Ewing sarcoma (ES) is the second most common bone and soft tissue sarcoma in young and adolescent population. Approximately 30% of ES cases present with metastatic disease at diagnosis. The most common sites for this malignancy are bones of lower extremity, pelvis and chest wall (ribs) (Burchill 2003, Jo and Fletcher 2014). The characteristic feature of ES is the presence of EWS-FLI1, a chimeric protein formed by the translocation between EWS and FLI1, in approximately 85% of cases (Zucman, Melot et al. 1993), while 10-15% of ES cases harbor variants of translocation between EWS and ERG genes (Delattre, Zucman et al. 1992).

The introduction of chemotherapeutic agents in 1960's was a paradigm shift in cancer treatment. However, chemotherapy used for ES is often associated with organ-specific side effects such as hormone imbalance and cardiotoxicity. Even though, EWS-FLI1 is known to be associated with ES, only limited progress has been made in the field of drug development tailored towards targeting this protein. Therefore, there is a need to identify alternate targets and less toxic therapeutic agents. Upregulation of transcription factors, proteins that regulate gene expression and control cellular homeostasis, are implicated in several cancers including sarcomas (Darnell 2002). Specificity protein 1 (Sp1) is ubiquitously expressed transcription factor which is critical in the regulation of cell cycle progression, cell proliferation, and apoptosis (Wang, Yang et al. 2011). Sp1 is considered as a prognostic factor for some cancers and targeting Sp1 can

provide a novel mechanism for cancer treatment (Beishline and Azizkhan-Clifford 2015, Hu, Hu et al. 2016, Liu, Ji et al. 2016).

Sp1 regulates the expression of several genes associated with cancer including survivin (Baculoviral inhibitor of apoptosis repeat containing 5) that belongs to Inhibitor of Apoptosis family. Survivin, regulates apoptosis and cell cycle in cancer cells. Overexpression of survivin has been linked to aggressiveness and poor prognosis in several cancers including sarcomas (Hingorani, Dickman et al. 2013) and its inhibition is reported to sensitize ES cells to radiation treatment (Greve, Sheikh-Mounessi et al. 2012). Disruption of survivin using siRNA has been shown to be effective in reducing tumor growth in pre-clinical models (Habib, Akhtar et al. 2015, Khan, Khan et al. 2016, Liu, Huang et al. 2016). Therefore, it is believed that targeting survivin provides a promising strategy for cancer treatment.

Mithramycin A (Mit) is an anti-neoplastic antibiotic isolated from *Streptomyces plicatus.* Mit has been shown to bind with GC rich DNA and inhibits transcription factors Sp1 and Sp3, and induces anti-cancer activity (Blume, Snyder et al. 1991). It is also evident that Mit downregulates survivin via modulating Sp transcription factors (Esteve, Chin et al. 2007). Several pre-clinical studies demonstrated similar activity of an anti-cancer non-steroidal anti-inflammatory drug (NSAID), Tolfenamic acid (TA) to target Sp proteins and survivin. We have previously investigated the anti-proliferative effect of TA in pediatric cancer models. These studies demonstrated that TA inhibits medulloblastoma and neuroblastoma cell growth by targeting Sp1 and survivin (Eslin, Lee et al. 2013, Eslin, Sankpal et al. 2013). TA has not been tested in pre-clinical/clinical models for human sarcomas.

In his study, we examined the anti-proliferative activity of TA in ES cells and the association of Sp1 and survivin in its anti-cancer activity using Mit as a positive control.

Initially, multiple ES cell lines were screened for the expression of Sp1 and survivin. Two selected ES cell lines were used for all subsequent assays. Knocking down of Sp1 using small interference RNA (siRNA) was used to test the significance of Sp1 in ES cell growth. Effect of TA on apoptosis in ES cells was studied by analyzing apoptosis by Annexin V staining. Expression of apoptosis markers, Caspase 3/7 activity and c-PARP proteins, was measured by Caspase 3/7-Glo kit and Western blot analysis respectively. propidium iodide (PI) flow cytometry staining was performed to evaluated the effect of TA on cell cycle phase distribution and, D type cyclins (Cyclin D1) protein expression was assayed using Western blot analysis. Both transcriptional and post-translational modifications were tested by evaluating mRNA expression and proteasome-dependent degradation using a proteasome inhibitor (Lactacystin), to understand the inhibitory effect of TA on Sp1 protein expression. The ability of Mit and TA to disrupt the Sp1 DNA-binding was also assessed using gel shift assay. This study tests the hypothesis that both Sp1 and survivin could serve as a reliable targets for inhibiting ES cell growth and identifies TA as a promising candidate to target Sp1 and survivin in ES cells.

MATERIAL AND METHODS

Cell culture

ES cell lines were obtained from the bio-repository of Children's Oncology Group (Lubbock, TX). CHLA-9, CHLA-10, TC-71, TC-32, CHLA-32 and CHLA-99 cells were grown in Iscove's Modified Dulbecco's Media (supplemented with 4mM L-Glutamine, 5 μg/mL

Insulin, 5 μg/mL Transferrin and 5 ng/mL Selenous Acid) and fetal bovine serum. Cells were maintained at 37°C and 5% CO_2.

Chemicals and Reagents

TA, Mit, dimethyl sulfoxide (DMSO), Lactacystin, and Anti-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Sp1 and Cyclin D1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and Survivin was purchased from R & D Systems (Minneapolis, MN). cleaved-PARP and Cyclin D3 antibodies were purchased from Cell Signal Technology (Danvers, MA). Dulbecco's phosphate-buffered saline was procured from Hyclone Laboratories (Logan, Utah). ITS premix was purchased from Corning (Bedford, MA). CellTiter-Glo kit and Caspase 3/7-Glo kit was procured from Promega (Madison, WI). Annexin V apoptosis kit was procured form BD Biosciences Ltd (San Jose, CA). Lipofectamine RNAi MAX reagent and Cell lysis buffer were procured from Invitrogen (Carlsbad, CA). Bicinchoninic acid (BCA) protein assay kit and Supersignal West Dura were purchased from Pierce (Rockford, IL).

Cell Viability Assay

Viability of CHLA-9 and TC-32 cells was assessed using CellTiter-Glo kit (Promega, Madison). Briefly, 4000 cells per well were seeded in 96-well plate and treated with DMSO (Control) or TA or Mit. After 24 or 48 h post-treatment, CellTiter-Glo reagent was added and luminescence was measured as per the manufacturer’s instructions using SYNERGY HT microplate reader (BioTek Instruments, Inc., Winooski, VT).

Caspase 3/7-Glo Assay:

CHLA-9 and TC-32 cells were treated with DMSO (control), TA (10 μg/ml) and TA (15 μg/ml), and the activity of caspase 3/7 was studied using Caspase-Glo 3/7 kit (Promega, Madison, WI), as per the manufacturer protocol. Briefly, 4,000 cells per well were seeded in a 96-well white walled clear bottom cell culture plate (Lonza, Basel, Switzerland). After 24 h of seeding, cell was treated with optimized doses of TA. 24h and 48 h post-treatment, cell viability (CellTiter-Glo) and Caspase 3/7 activity (Caspase 3/7-Glo) assays were performed. Luminescence was measured using SYNERGY HT microplate reader. Caspase 3/7 activity was normalized with cell viability readings.

Annexin V-PE Apoptosis Flow Cytometry Assay:

Apoptotic cell population was measured using Annexin V-PE apoptosis detection kit (BD Biosciences). Briefly, ES cells were with treated optimized doses of tolfenamic acid. 48 h post-treatment cells were incubated with Annexin V-PE antibody and 7-AAD for 15 min in 1X binding buffer. CHLA-9 and TC-32 cells were analyzed using Beckman Coulter FC500 flow cytometer and data was analyzed using FlowJo analysis software (Tree Star, Inc., Ashland, OR).

Propidium Iodide (PI) Cell Cycle Analysis

CHLA-9 and TC-32 cells were plated in 6 wells plates. Cells were treated with DMSO (control), TA (10 μg/ml) and TA (15 μg/ml). 24h and 48 h post-treatment cells were processed for cell cycle phase distribution analysis. Cells were washed with PBS and fixed in cold 70%

ethanol (prepared using PBS) for overnight at 4°C. Fixed cells were centrifuged and the cell pellets were washed with PBS and resuspended in propidium iodide (PI) buffer (0.20 µg/ml PI, 20 µg/ml RNAase A in PBS) and incubated for 15 min at room temperature, in dark. Cell cycle distribution was evaluated using Beckman Coulter FC 500 flow cytometer. Data was analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR) and represented as cell count vs. PI intensity (DNA content).

Western Immunoblot Analysis

CHLA-9 and TC-32 cells were plated in 10 cm culture dish and treated with DMSO or TA (10 or 15 µg/ml) or Mit (15 nM). After 24 or 48 h post-treatment, cells were harvested, protein extracts were prepared, and Western blot analysis was performed as described earlier (Eslin, Sankpal et al. 2013).

Small interfering RNA (siRNA) Transfection

CHLA-9 and TC-32 cells were transfected with 25 pmol of siRNA1 (catalogue no: J-026959-07) and siRNA2 (J-026959-08) Dharmacon (Lafayette, CO) that target Sp1, using Lipofectamine RNAi MAX reagent. Cells were harvested; 48 h post-transfection and whole cell lysates were prepared. Expression of Sp1 protein was determined using Western immunoblot analysis. Cell viability was measured with CellTiter-Glo assay kit as described for cell viability assay.

Quantitative Polymerase Chain Reaction (qPCR)

CHLA-9 and TC-32 were treated with DMSO (Control) or TA (15 μg/ml) or Mit (100 nM). Cells were harvested at 12 and 24 h post-treatment. RNA was extracted using TRIzol RNA extraction protocol (Invitrogen, Carlsbad, CA) and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The mRNA expression was evaluated by Roche thermal cycler using TaqMan primer-probes specific for Sp1 and Survivin purchased from Thermo Fisher (Waltham, MA).

Proteasome Inhibition Assay

CHLA-9 and TC-32 cells were treated with two doses of TA (10 μg/ml and 15 μg/ml) in the presence of 2 mM Lactacystin (proteasome inhibitor). 48 h post-treatment, cells were harvested. Whole cell lysates were prepared and the expression of Sp1 and β-Actin (loading control) was determined by Western blot analysis as described above.

DNA Binding Gel-shift Assay

Sp1 oligonucleotide (5'-ATTCGATCGGGGCGGGGCGAGC-3'-biotin; biotin-3'-TAAGCTAGCCCCGCCCCGCTCG-5') IDT ID: 1290543470 were obtained from Integrated DNA Technologies (Coralville, IA) and annealed to each other in annealing buffer. Binding reaction was carried out with TC-32 nuclear extract, binding buffer (10 mM Tris, 50 μM KCl and 10 nM DTT pH 7.5), and Sp1 oligo (biotin labelled) in the presence of DMSO (Control) or TA (50 μM) or Mit (50 μM) or positive control (100 μM of EDTA). Unlabeled oligo was used as a competitor. Sp1 oligo-protein complex was resolved on 6% polyacrylamide gel at 100 V and transferred to positively charged 0.2 μm nylon membrane using iBlot DNA transfer stacks (Invitrogen, Carlsbad, CA). The membranes were developed with Chemiluminescent Nucleic

Acid Detection Module Kit (Thermo Fisher, Waltham, MA) and the signal detected using UVP GelDoc imager.

Statistical Analysis

All experiments were performed at least in triplicates and the data were presented as mean ± SD (standard deviation). Data were analyzed using Graphpad Prism software V6.0 (La Jolla, California, USA) and following standard methods (student t-test or one-way ANOVA was performed; p value < 0.05 considered as statistical significance).

RESULTS

ES cell lines express Sp1 and survivin

The expression of key markers (Sp1, survivin) in ES cells was determined to understand their association with this malignancy. Several (six) ES cell lines were screened by Western immunoblot to examine the expression of Sp1 and survivin. All cell lines tested expressed Sp1 and survivin (Figure 3.1). Interestingly the expression of Sp1 and survivin show similar pattern suggesting a correlation between these candidates.

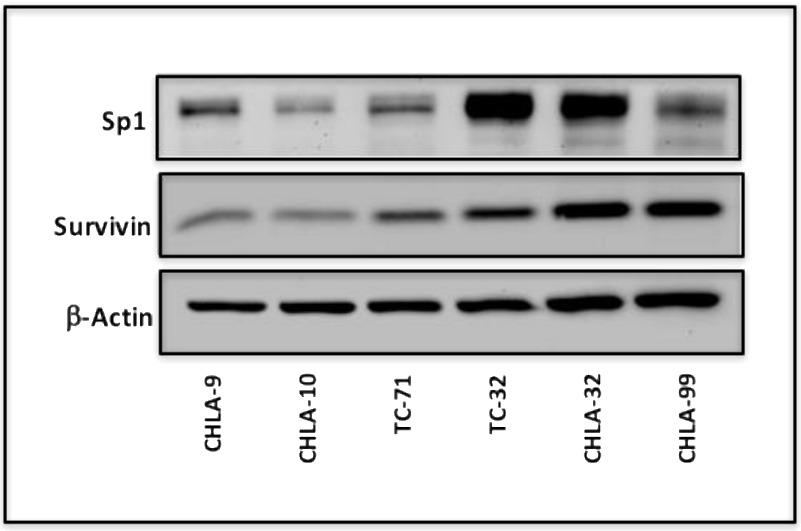


Figure 3.1: Sp1 and survivin expression in ES cells. Whole cell lysates were prepared from ES cell lines CHLA-9, CHLA-10, TC-71, TC-32, CHLA-32 and CHLA-99. Expression of Sp1 and survivin was determined using Western blotting. β-Actin expression is shown as a loading control.

Sp1 knockdown inhibits cell viability of ES cells

In order to study the effect of Sp1 inhibition on ES cell growth, we performed siRNA knockdown of Sp1 transcription factor in ES cells and measured the cell viability at concurrent

time-point. Results showed that both the siRNAs (siRNA1 and siRNA2) inhibited the expression of Sp1 protein than control. The inhibition of Sp1 strongly correlated with a decrease in cell viability in both CHLA-9 and TC-32 cell lines transfected with siRNAs (Figure 3.2).

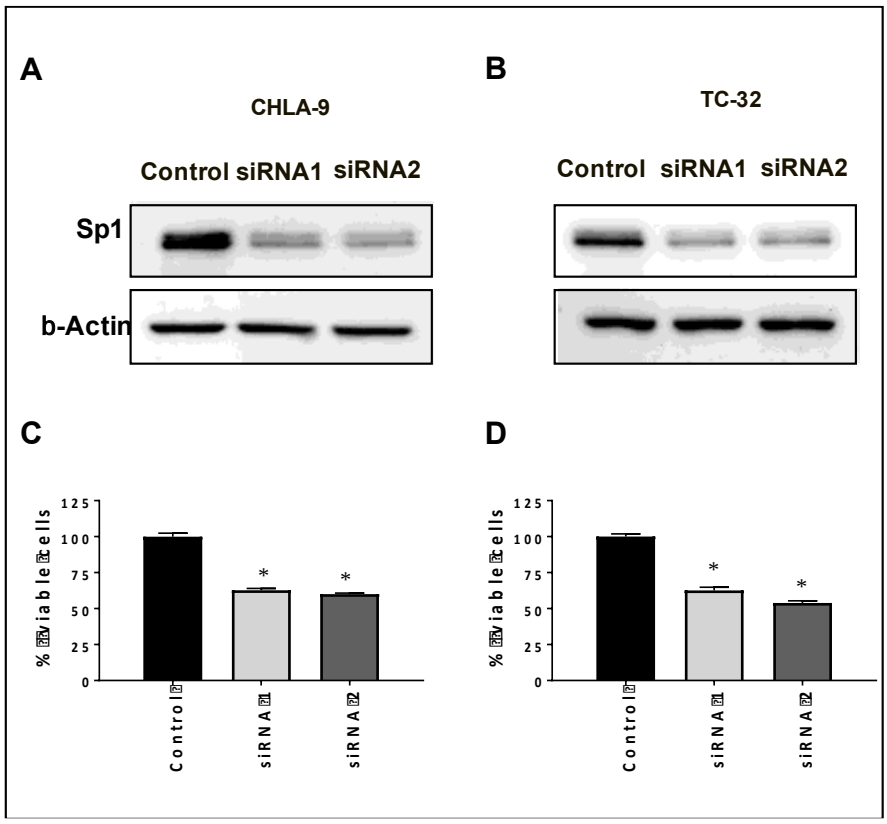


Figure 3.2: Sp1 silencing using siRNA inhibits Sp1 protein expression and decreases cell viability in ES cells. A) CHLA-9 and B) TC-32 cells were transfected with two different Sp1 siRNAs. 48 h post-transfection Sp1 protein expression was determined by Western immunoblotting. β-Actin was used as a loading control. C) CHLA-9 and D) TC-32 cells were transfected with siRNA 1 and siRNA 2 specific for Sp1. After 48 h cell viability assay was performed using CellTiter-Glo reagent. One-way ANOVA test was performed for statistical analysis. Bars represent the mean ± SD of three independent determinations. * represent *p*-value <0.05.

TA and Mit treatment inhibited cell viability of ES cells

The effect of TA and Mit (positive control) on the proliferation of CHLA-9 and TC-32 cells was evaluated. Cells were treated with TA (0-30 µg/ml) and cell viability was measured at 24 & 48 h post-treatment. TA significantly inhibited cell viability following a clear dose/ time-dependent pattern in both CHLA-9 and TC-32 cells. The IC50 values at 48 h were ranging from 11-15 µg/ml (CHLA-9: 11.26 µg/ml; TC-32: 14.79 µg/ml) for TA as calculated by nonlinear curve fitting (Figure 3.3).

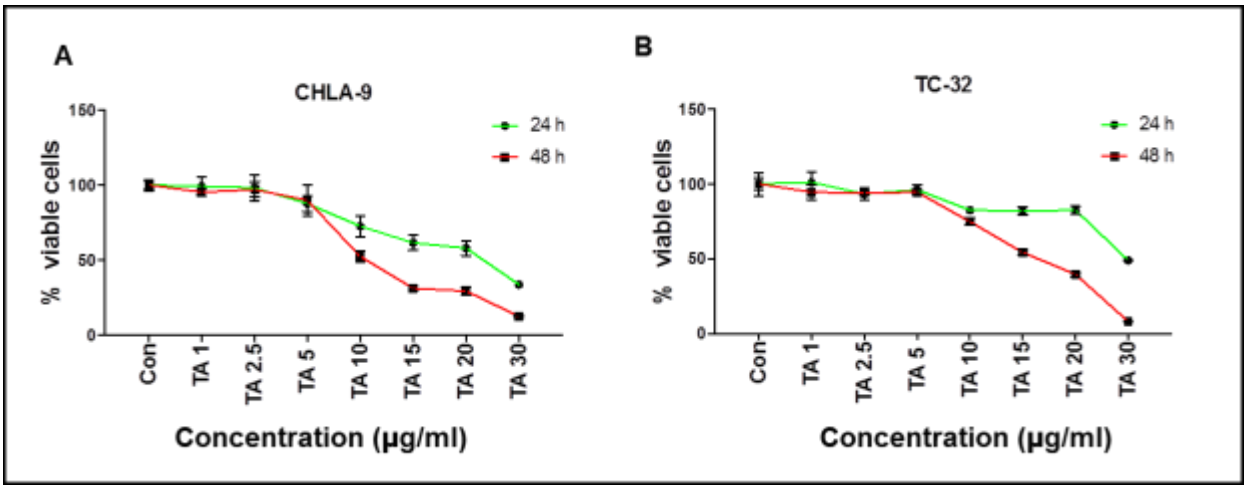


Figure 3.3: TA inhibited cell viability of ES cells. A) CHLA-9 and B) TC-32 cells were treated with DMSO (Control) or (0-30 µg/ml) of TA. Cell viability was assessed at 24 and 48 h post-treatment using CellTiter-Glo assay kit. Each data point on the line represents mean ± SD of triplicate determinations.

TA inhibited Sp1 and survivin protein expression in ES cells

CHLA-9 and TC-32 cells were treated with vehicle (DMSO), TA (10 µg/ml) or TA (15 µg/ml) or Mit (15 nM) and cells were harvested at 24 and 48 h post-treatment. Sp1 and survivin protein

expression was determined by Western immunoblot analysis. TA treatment significantly decreased the Sp1 and survivin in dose- and time- dependent manner in both CHLA-9 and TC-32 cells (Figure 3.4). As expected, results showed that Mit also decreased expression of Sp1 and survivin in both (tested) cell lines (Shelake, Sankpal et al. 2016).

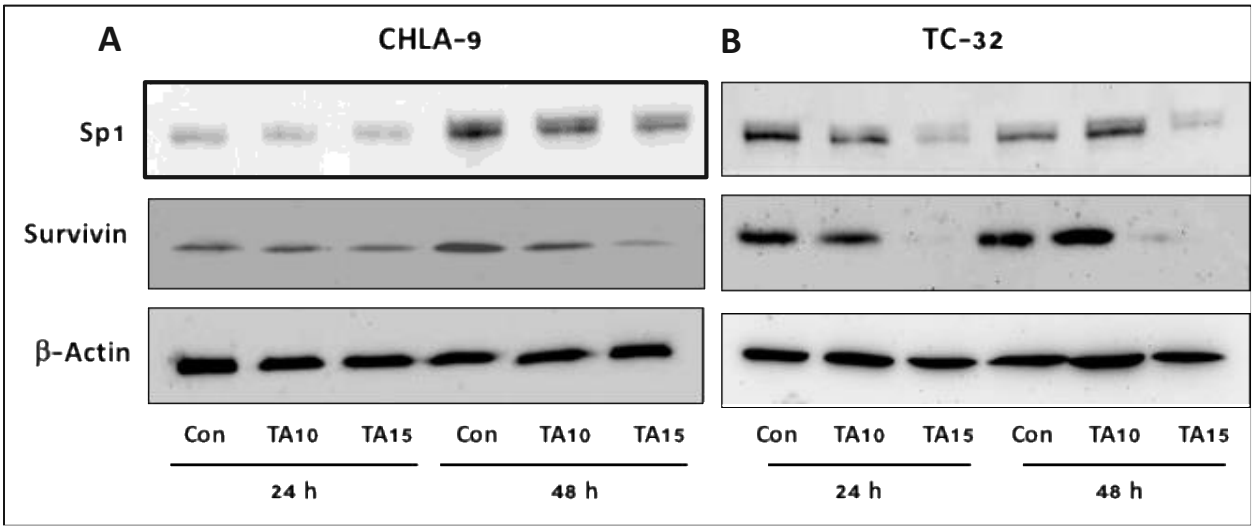


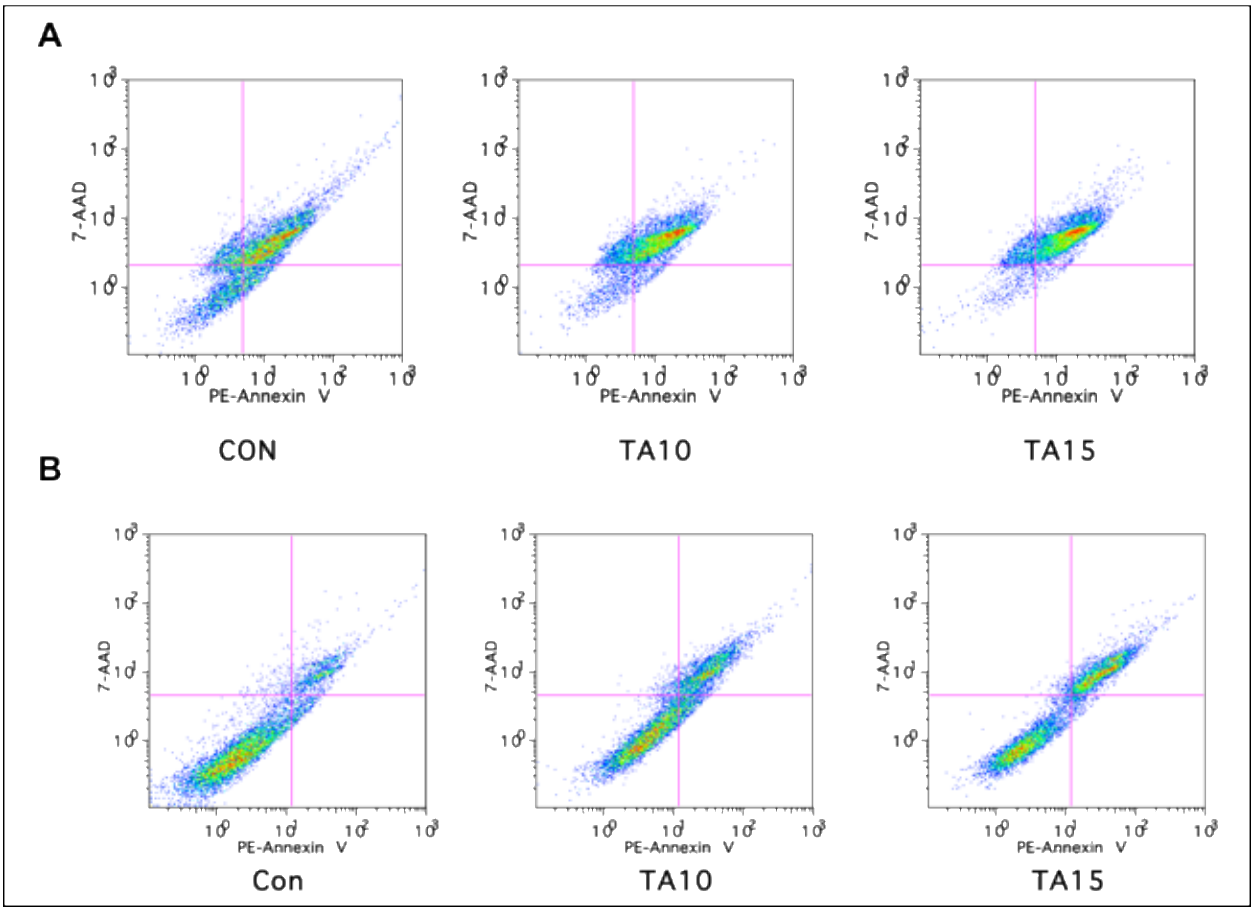
Figure 3.4: TA treatment decreased Sp1 and survivin protein expression. A) CHLA-9 and B) TC-32 cells were treated with DMSO, TA (10 μg/ml) or TA (15 μg/ml). At 24 and 48 h post-treatment cells were harvested and Sp1 and survivin protein expression was assayed (Western immunoblot analysis). β-Actin was used as a loading control.

Con: Control (DMSO treated); TA10: Tolfenamic acid, 10μg/ml; TA15: Tolfenamic acid, 15μg/ml.

TA treatment induced apoptosis in ES cells.

The effect of TA induced apoptosis in ES cells was determined by evaluating caspase 3/7 activity and c-PARP protein expression. CHLA-9 and TC-32 cells were treated with vehicle control (DMSO) and two optimized doses of TA (10 μg/ml and 15 μg/ml). 48 h post-treatment

Annexin V staining was performed and data was analyzed with flow cytometry. Results revealed that TA treatment increased Annexin-V staining (CHAL-9: TA10=1.1 fold, TA15=1.4 fold; TC-32: TA10=2.4 fold, TA15= 3.2 fold) (Figure 3.5).



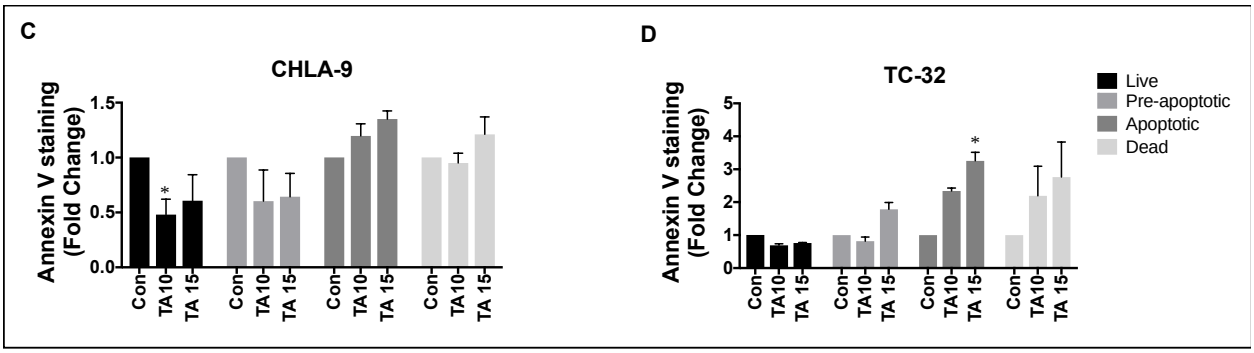


Figure 3.5: Tolfenamic acid treatment increased Annexin V apoptotic cell population ES cells. A, B) CHLA-9 and C, D) TC-32 cells were treated with DMSO, TA (10 μg/ml) or TA (15 μg/ml) Annexin V staining were performed at 48 h post-treatment. Bars represent mean ± SD of data obtained from three independent experiments. Representative Annexin-V graphs are shown. Statistical analysis performed by one-way ANOVA using Graphpad Prism V6.0. * represents *p* value <0.05.

Con: Control (DMSO treated); TA10: Tolfenamic acid, 10 μg/ml; TA15: Tolfenamic acid, 15 μg/ml.

In order to investigate the underlying signaling pathway, Caspase 3/7 activity was measured using Caspase 3/7-Glo kit and normalized to cell viability results. c-PARP protein expression was measured using Western blot analysis. At 48 h post-treatment, TA10 treatment (CHLA-9: 1.8 fold; TC-32: 2.6 fold) and TA15 treatment (CHLA-9: 3.1fold; TC-32: 2.4 fold) significantly increased Caspase 3/7 activity compared to the respective control. Consistent with these results, TA treatment increased c-PARP protein expression in time- and dose- dependent manner in ES cells (Figure 3.6).

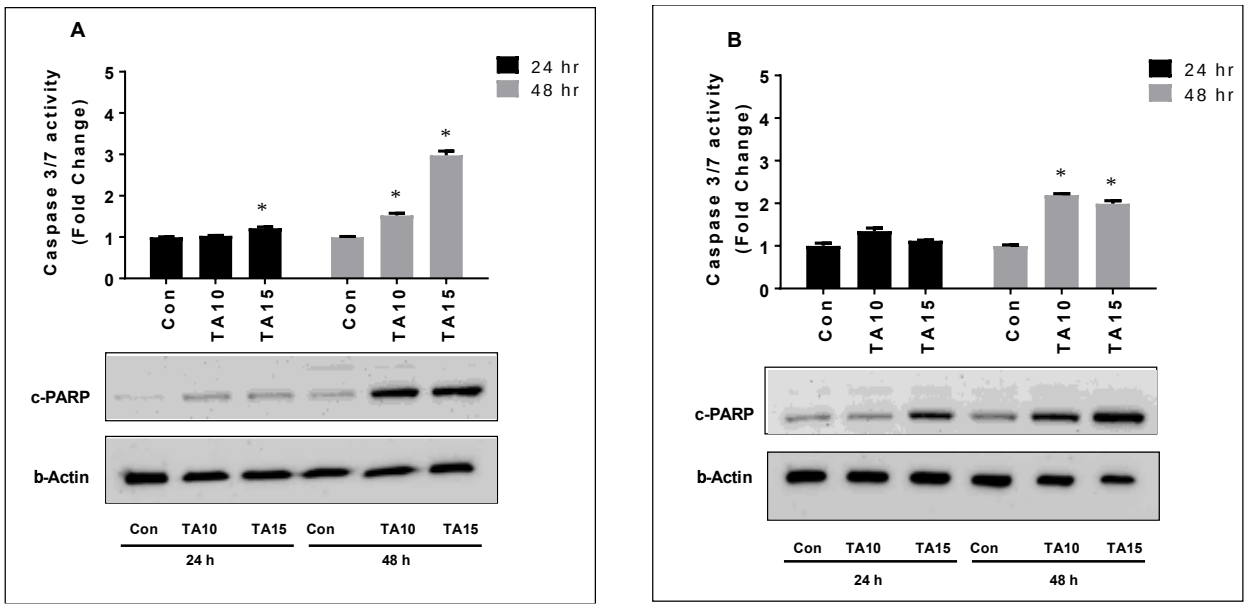


Figure 3.6: Tolfenamic acid increased Caspase 3/7 activity and c-PARP level in dose- and time- dependent manner. A) CHLA-9 and B) TC-32 cells were treated with DMSO, TA (10 μg/ml) or TA (15 μg/ml) Caspase 3/7 activity was measured using caspase 3/7 Glo kit at 24 h and 48 h post-treatment. Whole Cell lysates were prepared and the expression of c-PARP was evaluated by Western blot analysis at 24 h and 48 h post-treatment. The data were obtained from at least three independent determinations and representative gels are shown in the figure. All data represent the mean ± SD. Statistical analysis performed by one-way ANOVA using Graphpad Prism V6.0. * represents *p* value <0.05.

Con: Control (DMSO treated); TA10: Tolfenamic acid, 10 μg/ml; TA15: Tolfenamic acid, 15 μg/ml.

TA treatment resulted in G0/G1 cell cycle arrest in ES cells.

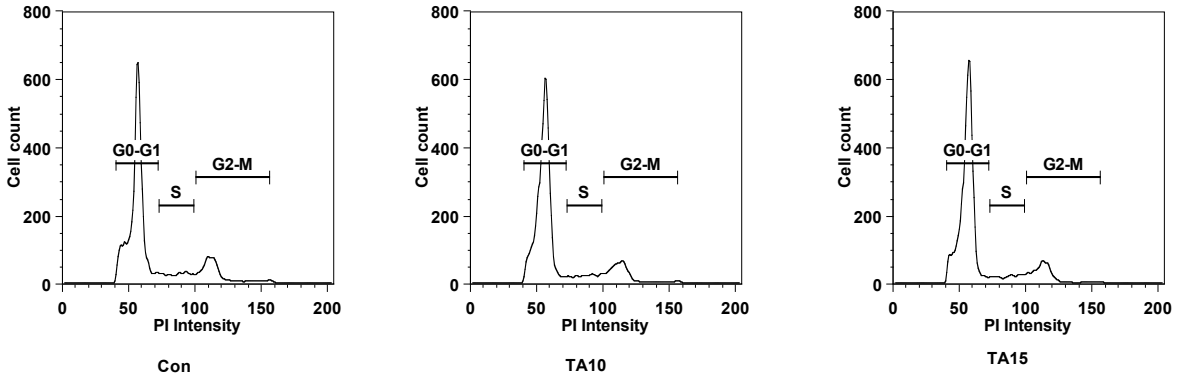
TA caused significant inhibition of cell viability in a dose- and time-dependent manner in ES cells and increased apoptosis markers. Previously, we have showed that TA treatment causes G0/G1 cell cycle arrest accompanied by increased apoptosis in neuroblastoma cells (Eslin,

Sankpal et al. 2013). Thus, to evaluate whether TA induces its anti-proliferative in similar mechanism, we assayed cell cycle phase distribution in ES cell using flow cytometric analysis. CHLA-9 and TC-32 cells were treated with vehicle control (DMSO) and two optimized doses of TA (10 μg/ml and 15 μg/ml). Our results showed that at 48 h post-treatment TA 15 μg/ml significantly increased G0/G1 cell cycle arrest (77.7 ± 0.2%) compared to control (71.8±0.2%) in CHLA-9. However, at 24 h such effect was not observed in CHLA-9 cells (Figure 3.7A & 3.7B). In contrast, both TA 10 μg /ml and TA 15 μg/ml doses significant increased G0/G1 cell cycle arrest in TC-32 cells than control. This increase in G0/G1 cell cycle arrest in TC-32 cells was higher at 24 h (TA10: 64.7 ± 0.4%; TA15: 72.4 ± 0.6%) as compared to 48 h post-treatment (TA10: 63.2 ± 1.9%; TA15: 68.2 ± 0.3%) (Figure 3.7C & 3.7D).

In order to determine the signaling pathway involved in the regulation of cell cycle phase distribution, we studied D type cyclins that are important in modulation of cell cycle phase transition from G1 to S phase. Previously, pharmacological inhibition of cyclin D1 alone is shown to induce G0/G1 cell cycle arrest in ovarian cancer cells (Masamha and Benbrook 2009). Importantly Cyclin D1 is upregulated in ES clinical specimens and is linked to poor prognosis of the ES (Fagone, Nicoletti et al. 2015, Magro, Brancato et al. 2015). Our results showed that, TA 15 μg/ml treatment decreased the Cyclin D1 protein expression and it strongly correlated with G0/G1 cell cycle phase arrest observed on ES cells at 48 h post-treatment (Figure 3.8).

A

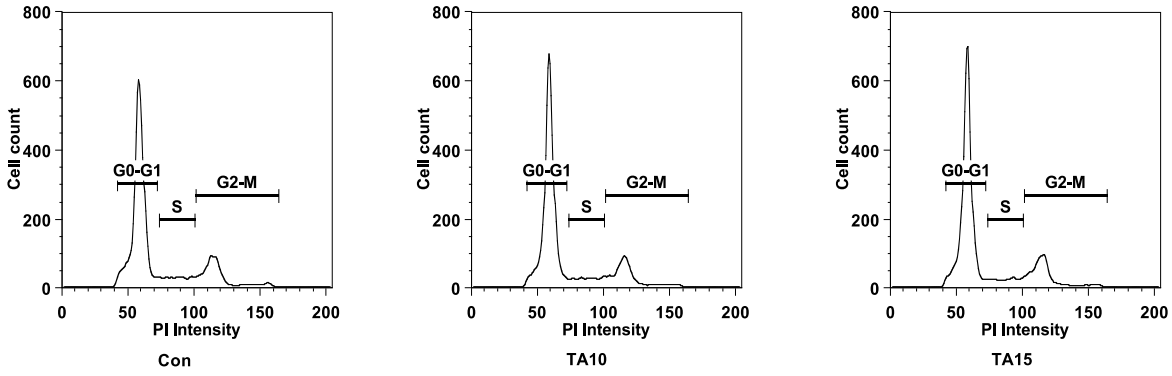
CHLA-9 (24 h)



	Con	TA10	TA15
G0/G1	68.97±0.68	70.50±1.21	70.60±0.56
S	9.94±0.30	8.62±0.34	7.93±0.77
G2/M	19.80±0.98	19.83±1.62	20.50±1.13

B

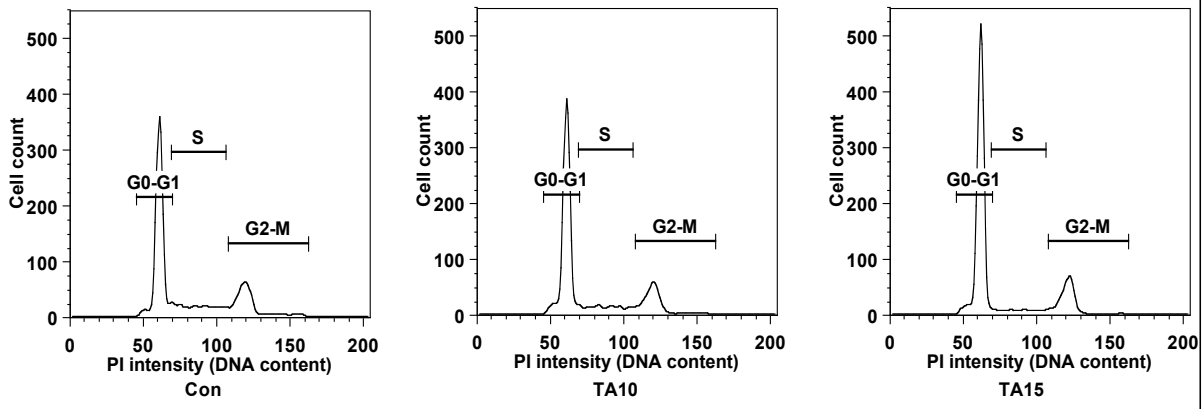
CHLA-9 (48 h)



	Con	TA10	TA15
G0/G1	71.83±0.32	76.73±0.70	77.47±0.38 *
S	9.32±0.29	7.45±0.23	7.10±0.23
G2/M	17.43±0.06	14.80±0.40	14.40±0.36

C

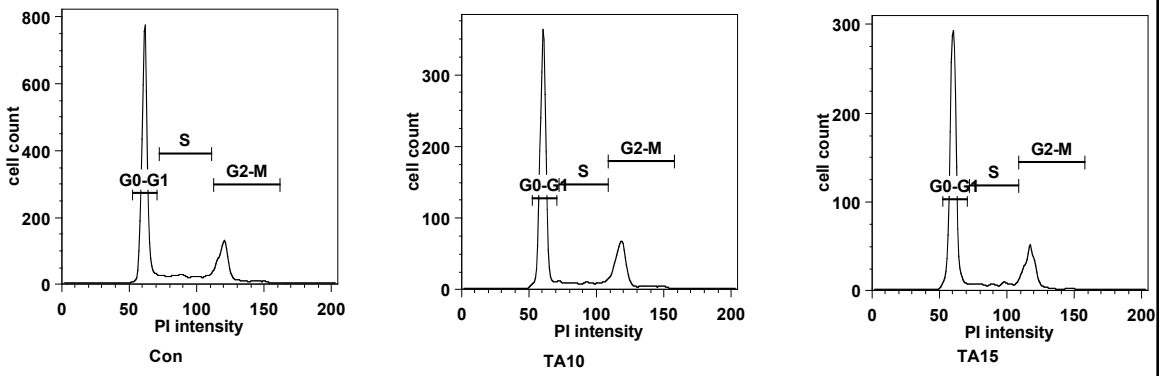
TC-32 (24 h)



	Con	TA10	TA15
G0/G1	55.97±1.99	64.70±0.62 (P< 0.05)	72.37±1.04*
S	19.00±0.70	12.30±1.75	8.07±0.61
G2/M	25.20±1.47	22.93±1.91	19.50±1.06

D

TC-32 (48 h)



	Con	TA10	TA15
G0/G1	62.70±0.46	63.20±3.27	68.17±0.55*
S	13.27±0.40	10.00±0.28	9.43±0.80
G2/M	23.37±0.68	26.17±3.37	21.73±1.01

Figure 3.7: TA treatment caused G0/G1 call cycle arrest in ES cells. A, B) CHLA-9 and C, D) TC-32 cells were treated with DMSO or TA (10 μg/ml) or TA (15 μg/ml). TA 24 and 48 h post-treatment cells were harvested and cell cycle phase distribution was assessed using PI cell cycle staining analysis. The data were obtained from three independent experiments. Representative graphs are shown in the figure. All data represent the mean ± SD. Statistical analysis performed by two-way ANOVA using GraphPad Prism V6.0 (* $p<0.05$).

Con: Control (DMSO treated); TA10: Tolfenamic acid, 10 μg/ml; TA15: Tolfenamic acid, 15 μg/ml.

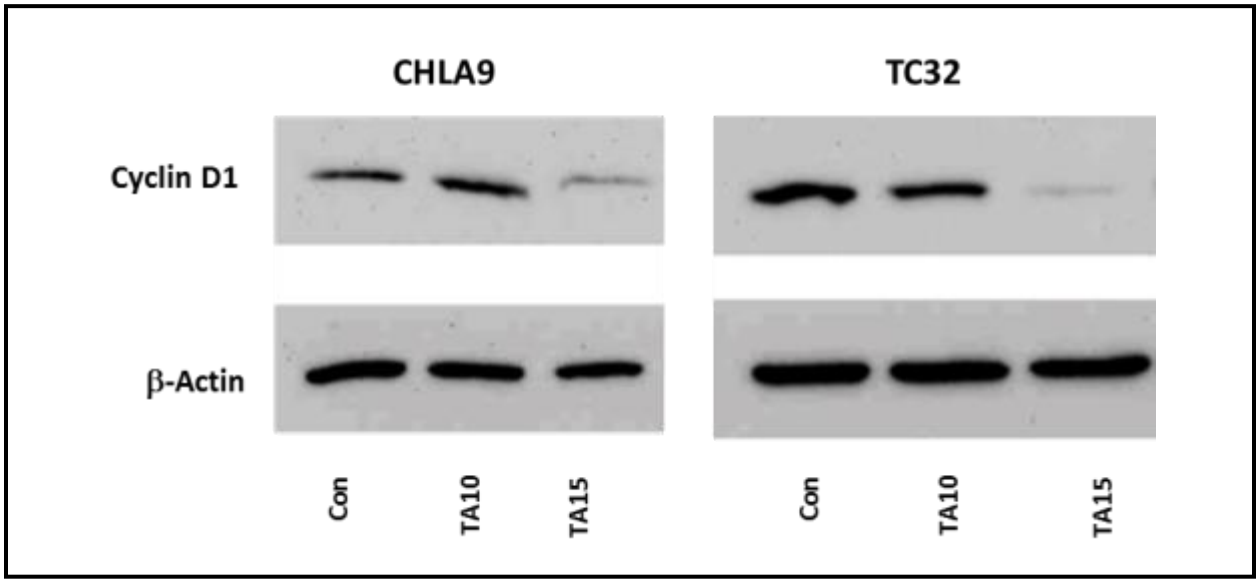


Figure 3.8: TA treatment decreased Cyclin D1 protein expression. A) CHLA-9 and B) TC-32 cells were treated with DMSO or TA (10 μg/ml) or TA (15 μg/ml). At 48 h posttreatment cells were harvested and Cyclin D1 protein expression was assayed by Western immunoblot analysis. β-Actin was used as a loading control.

Con: Control (DMSO treated); TA10: Tolfenamic acid, 10 μg/ml; TA15: Tolfenamic acid, 15 μg/ml.

Effect of TA and Mit on Sp1 and survivin mRNA expression

Levels of Sp1 and survivin mRNA were evaluated in CHLA-9 and TC-32 cells following 12 and 24 h treatment with Mit (100 nM) or TA (15 μg/ml) using qPCR. Mit treatment significant decreased Sp1 and survivin mRNA expression in both cell lines at 24 h post-treatment (Figure 9.3 A&B). TA inhibited survivin mRNA expression at 24 h post-treatment; however, it did not change Sp1 mRNA expression in ES cells (Figure 3.9 C&D).

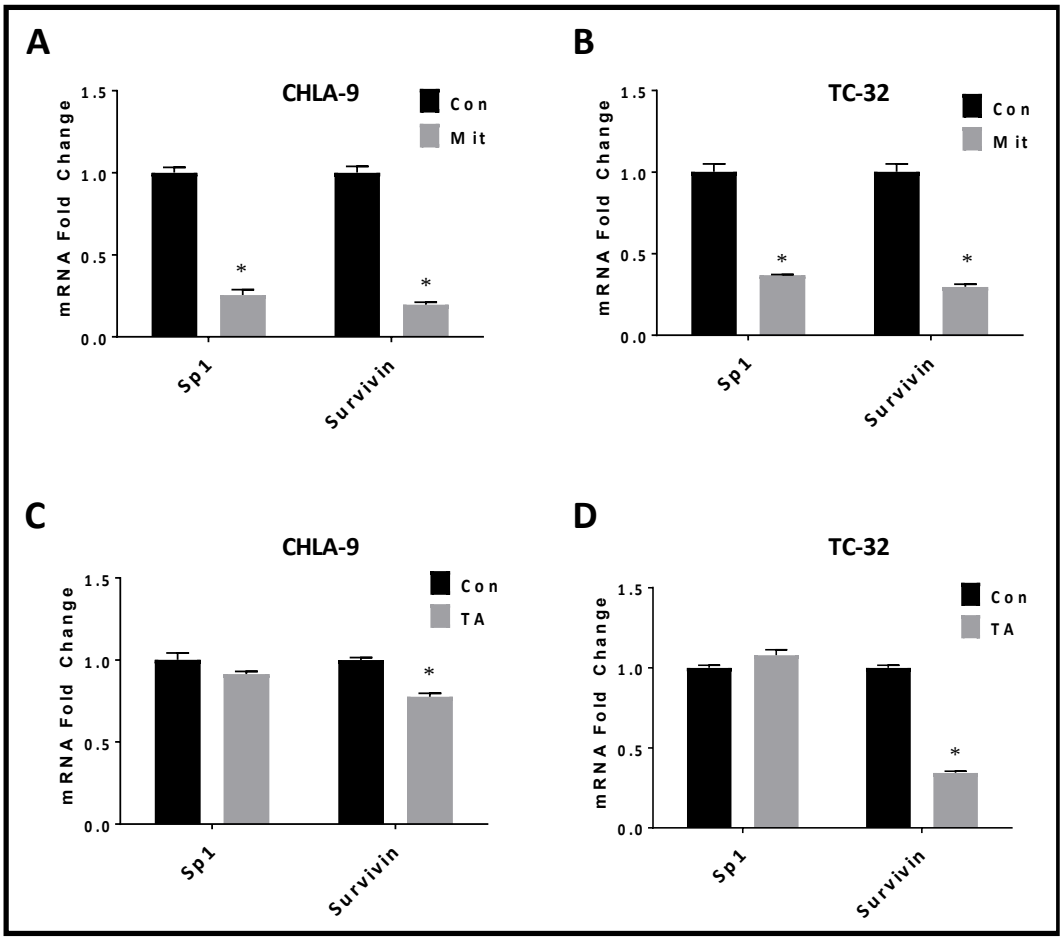


Figure 3.9: Effect of TA and Mit treatment on Sp1 and survivin mRNA. A, C) CHLA-9 and B, D) TC-32 cells were treated with DMSO, TA (15 μg/ml) or Mit (100 nM). Total RNA was

isolated with TRIzol RNA extraction protocol. Sp1 and survivin mRNA expression was measured using qPCR. GAPDH was used as loading control Student-t test was performed for statistical analysis. Bars represent mean ± SD of three independent replicates. "*" represents p value <0.05.

TA induces proteasome-dependent Sp1 degradation

Previously it was reported that TA induced the activation of proteasome dependent degradation of Sp1 in pancreatic cancer cell lines (Abdelrahim, Baker et al. 2006) but not in esophageal cancer cells (Papineni, Chintharlapalli et al. 2009). In this study, we investigated whether TA treatment induces the activation of proteasome-mediated Sp1 degradation in ES cells. CHLA-9 and TC-32 cells were treated with vehicle (DMSO) or TA in the presence or absence of Lactacyctin, a proteasome inhibitor. Cells were harvested at 48 h and whole cell lysates were prepared. Results revealed that Lactacystin treatment caused modest restoration of TA-induced inhibition of Sp1 protein expression in both CHLA-9 and TC-32 ES cells (Figure 3.10).

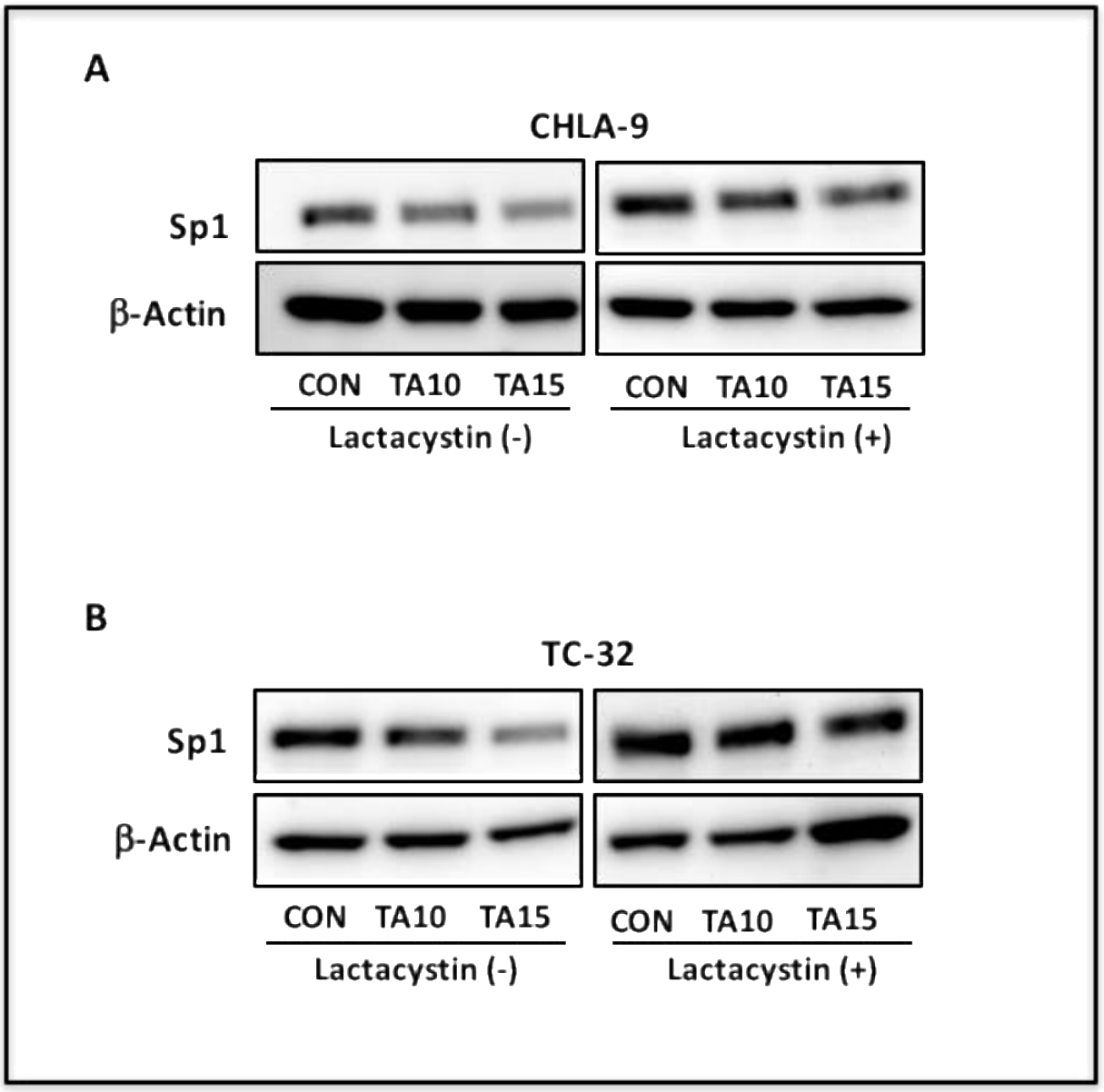


Figure 3.10: Lactacystin blocked TA induced proteasome-dependent degradation of Sp1 protein. A) CHLA-9 and B) TC-32 cells were treated with DMSO or TA (15 μg/ml) or Lactacystin (2 mM) + TA (15 μg/ml). Cells were harvested at 48 h post-treatment and Sp1 and β-Actin (loading control) protein expression was analyzed by Western immunoblot. β-Actin was used as a loading control.

Con: Control (DMSO treated); TA10: Tolfenamic acid, 10 µg/ml; TA15: Tolfenamic acid, 15 µg/ml.

TA disrupted the DNA-binding activity of Sp1

Since Sp1 is a transcription factor, its ability to bind to DNA is critical for its functional activity. We tested the ability of Sp1 to bind to the Sp1 consensus oligo, in the presence of TA or positive control (Mit), by gel shift assay. ES cell nuclear extract was used as the source for Sp1 protein. The gel shift assay results demonstrated that both Mit (Shelake, Sankpal et al. 2016) and TA inhibited the Sp1 DNA-binding, consistent with the positive control (EDTA) (Figure 3.11). The specificity of Sp1 DNA-binding was confirmed by the competition assay, where the addition of excess unlabeled Sp1 oligo competed with biotin labeled oligo and completely knocked down the binding of Sp1 (from ES cell nuclear extract) to biotin labeled oligo (Figure 3.11).

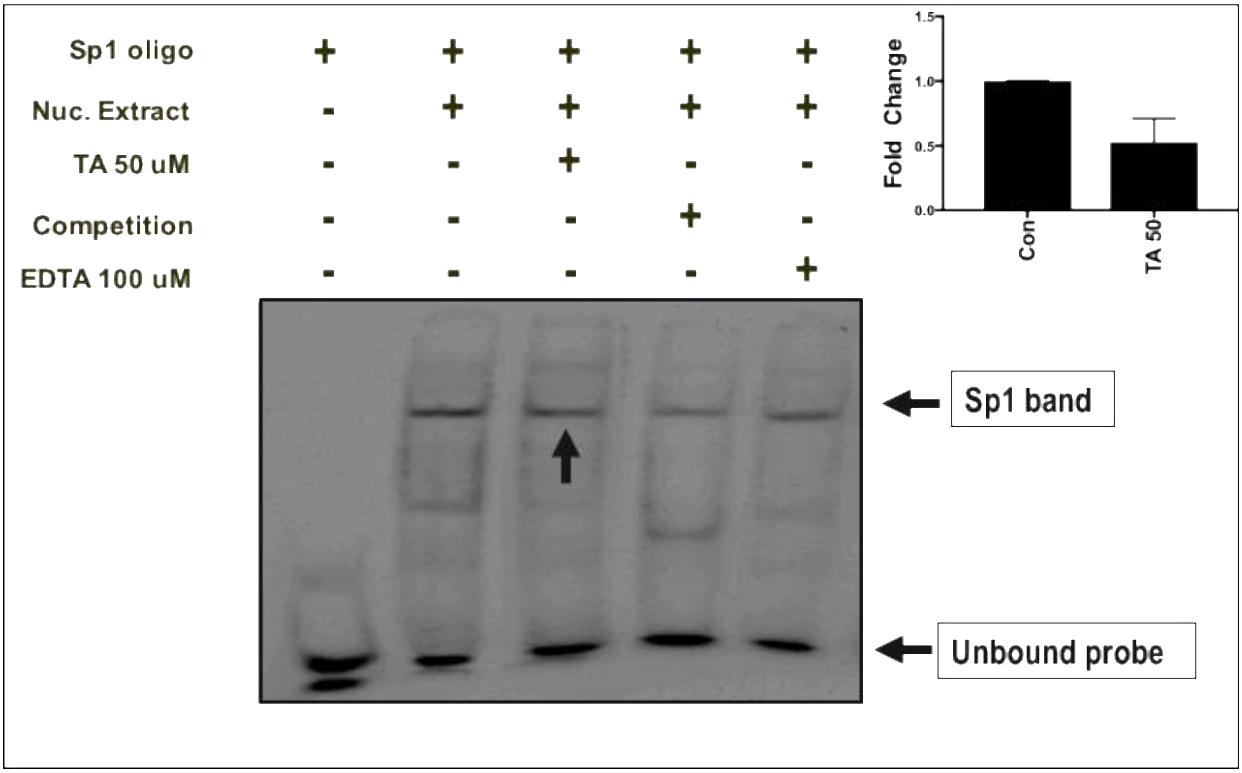


Figure 3.11: TA inhibited DNA-binding activity of Sp1 transcription factor. Sp1 DNA-binding assay was performed with TC-32 nuclear extract and biotin labeled Sp1 oligo. Nuclear extracts were incubated with TA (50 μM) or EDTA (100 nM) or 10X concentration of unlabeled Sp1 oligo (competitor) for 15 min. and gel shift assay was performed to examine perturbations in the Sp1 DNA-binding. Sp1 band intensities of control and TA 50 treatment samples were quantified using NIH ImageJ software.

DISCUSSION

Sp1 is one of the first identified mammalian transcription factors. It is important for the regulation of critical cellular processes such as cell cycle progression and apoptosis and impacts cell proliferation. Studies have also demonstrated the regulation of cell proliferation and apoptosis by Sp1 via modulating the expression of survivin, vascular endothelial growth factor and cyclin D1 (Fuchs, Inwards et al. 2004, Giorgi, Boro et al. 2015). Although Sp1 is expressed in ES and viewed as ‘’hallmark of cancer”, the strategies to target Sp1 are still lacking (Beishline and Azizkhan-Clifford 2015). High expression of survivin is associated with aggressiveness and poor prognosis in various cancer types including ES (Fukuda and Pelus 2006, Hingorani, Dickman et al. 2013). In this study we investigated the anti-proliferative activity of TA and Mit in association with their efficacy to target Sp1 and survivin in ES cells.

Sp1 and survivin are considered as prognostic markers in several malignancies (Greve, Sheikh-Mounessi et al. 2012, Hingorani, Dickman et al. 2013, Beishline and Azizkhan-Clifford 2015, Habib, Akhtar et al. 2015, Hu, Hu et al. 2016, Khan, Khan et al. 2016, Liu, Ji et al. 2016, Liu, Huang et al. 2016), and recently the association of these candidates in ES is under investigation (Yan, Li et al. 2013, Giorgi, Boro et al. 2015). In this study, six ES cell lines obtained from COG were screened to evaluate the presence of Sp1 and survivin and all six cell lines showed moderate to high levels of expression for both Sp1 and survivin (Figure 3.1). Silencing of Sp1 in CHLA-9 and TC-32 cells using siRNA technology resulted in significant decrease in cell viability correlating with the inhibition of Sp1 protein levels (Figure 3.2). These results strongly suggest that inhibition of Sp1 can induce an anti-proliferative response in ES cells. Mit is an antibiotic with antineoplastic properties which was shown to target Sp proteins and inhibit cell proliferation and tumor growth in some pre-clinical models (Wang, Guan et al.

2008, Choi, Jung et al. 2013, Shin, Jung et al. 2013). TA also showed similar effects and inhibited cancer cell growth by inhibiting Sp1 and survivin protein expression in preclinical studies (Abdelrahim, Baker et al. 2006, Konduri, Colon et al. 2009).

Our results suggest that the anti-proliferative effect seen in TA treatment could be partially due activation of apoptosis pathways. To study the effect of TA on cell apoptosis, we performed the flow cytometric Annexin V assay. Results showed that TA caused several fold increase in (CHAL-9: TA10=1.1 fold, TA15=1.4 fold; TC-32: TA10=2.4 fold, TA15= 3.2 fold) Annexin-V cell staining in ES cells (Figure 3.5). In order to determine the signaling pathway involved behind the apoptosis mechanism, we measured Caspase 3/7 activity by Caspase 3/7-Glo assay and cleaved PARP protein expression by Western blot analysis. Consistent with Annexin V flow cytometry results, TA increased Caspase 3/7 activity in a time- and dose- dependent manner that strongly correlated with increase in c-PARP protein level (Figure 3.6) and decreased cell viability. Survivin is known to inhibit effector caspases, mainly Caspase 3/7 (Grossman, Kim et al. 2001, Obexer, Hagenbuchner et al. 2009, Xie, Liu et al. 2012). Therefore, TA induced inhibition of survivin could be resulting in dramatic activation of Caspase 3/7.

Our laboratory has shown that TA induces anti-proliferative effect in Neuroblastoma cells by causing G0/G1 cell cycle arrest (Eslin, Sankpal et al. 2013). Amplification or overexpression of D type cyclins (such as Cyclin D1 and Cyclin D3) is reported to alter G0/G1 to S phase cell cycle transition, and shown to play critical role in human malignancies including breast, melanoma, prostate and lymphoma. Recently, Cyclin D1 is reported to be overexpressed in ES clinical specimens and is linked to poor prognosis in ES patients (Fagone, Nicoletti et al. 2015, Magro, Brancato et al. 2015). We hypothesized that TA could be causing its anti-proliferative effect in ES cell by interfering with cell cycle phase distribution in ES cells. We

performed a PI cell cycle analysis to investigate the effect of TA on cell cycle in ES cells. Results revealed that TA 15 μg/ml treatment induced significant increase in G0/G1 cell in ES cells at 48 h. TA caused G0/G1 cell cycle in TC-32 cells was higher at 24 h than 48 h. It is possible that TA could be causing anti-proliferative activity primarily via cell cycle arrest, and is proceeds activation of apoptosis pathway in TC-32 cells (Figure 3.7). The effect of TA on Cyclin D1 protein expression was determined by Western blot analysis. Results showed that TA 15 μg/ml treatment inhibited Cyclin D1 protein expression at 48 h, and that this inhibition strongly correlated with G0/G1 arrest observed in PI cell cycle analysis (Figure 3.8).

TA treatment caused a time/dose-dependent decrease in cell viability (Figure 3) that was accompanied by a decrease in Sp1 and survivin protein as determined by Western immunoblot (Figure 3.4). Both TA and Mit have been shown to inhibit Sp1 protein expression in several cancers, hence the growth inhibition of ES cells by TA and Mit could be attributed to the effect of these agents on Sp1 and survivin. We also investigated the effect of Mit and TA on mRNA expression of Sp1 and survivin. Both TA and Mit treatment caused significant inhibition of survivin mRNA expression (Figure 3.9). Mit significantly decreased the mRNA expression of Sp1 (Figure 3.9A & 3.9B), however, TA did not cause any change in Sp1 expression levels (Figure 3.9C & 3.9D) suggesting involvement of post-translational effects. Lactacystin, a proteasome inhibitor binds and blocks the 20S subunit of proteasome complex. In this study, Lactacystin blocked TA-induced Sp1 inhibition (Figure 3.10) confirming the proteasome-dependent degradation of Sp1 by TA. These results are in agreement with an earlier study which demonstrated that TA caused proteasome-dependent degradation of Sp1 in pancreatic cancer cell lines (Abdelrahim, Baker et al. 2006).

Gel shift assay revealed that the both Mit and TA caused perturbations in DNA-binding activity of Sp1 (Figure 3.11) and published results (Shelake, Sankpal et al. 2016). It is plausible that TA could be working as a chelating agent disrupting the zinc finger motif thereby decreasing the DNA-binding activity of Sp1 to its consensus sequence (Figure 3.11). Therefore, Mit and TA could affect the activity of Sp1 and potentially impact the expression of genes that are regulated by Sp1.

Survivin is implicated in the pathogenesis of several cancers including sarcomas. Sp1 transcription factor regulates survivin and also known be associated with cancer. Both of these candidates are linked to poor prognosis and considered as potential targets in cancer treatment. However, there are no specific agents identified until now to specifically target Sp1 and/or survivin. In this investigation, we report that the

small molecule (NSAID), tolfenamic acid causes anti-proliferative response in ES cells. Tolfenamic acid induces cell apoptosis, increases apoptosis markers and causes cell cycle arrest potentially through inhibiting Sp1 and survivin protein expression. We demonstrate that inhibition of Sp1 transcription factor using TA and Mit effectively decreased CHLA-9 and TC-32 cell growth. Mechanistically, we also show that inhibiting Sp1 resulted in inhibition of survivin protein and mRNA expression. At molecular level both TA and Mit treatment significantly inhibited Sp1 DNA-binding activity. We also show that TA induced activation of proteasome-dependent Sp1 degradation, whereas Mit treatment caused transcriptional inhibition of Sp1. Overall, results of this study demonstrate that ES cells express Sp1 and survivin, and TA and Mit significantly decreased ES cell viability which correlated with an inhibition of Sp1 and survivin protein expression; however, TA and Mit vary in their mechanism of action to modulate Sp1 activity.

EWS-FLI1 is overexpressed in majority of ES tumors. High throughput screening identified Mit as an effective agent to target EWS-FLI1 (Grohar, Woldemichael et al. 2011) and Federal Drug Administration (FDA) approved its testing on ES patients. Since there were some concerns on Mit side effects, it is important to explore alternative targets and less toxic agents for clinical testing. Non-steroidal anti-inflammatory drugs are being widely tested for their anti-cancer activity (Jacoby, Seibert et al. 2000, Koki and Masferrer 2002, Tarnawski and Jones 2003, Gately and Li 2004, Juni, Reichenbach et al. 2005). TA has been used for treating migraine headaches and well tolerated by patients. It is a well-studied NSAID with limited toxicity among several other commonly used NSAIDs such as ibuprofen. Therefore, testing this agent will serve as a robust and cost-effective strategy for the diseases like ES. Collectively, our results provide evidence for the significance of targeting Sp1 transcription factor and survivin as therapeutic targets for the treatment of ES and suggest that TA could serve as an effective agent.

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CHAPTER IV

TOLFENAMIC ACID ENHANCES ANTI-PROLIFERATIVE EFFECT OF VINCRISTINE IN EWING SARCOMA CELL LINES.

ABSTRACT

Vincristine (Vin), a Vinca alkaloid derived from periwinkle plant, is an integral part of many chemotherapeutic regimens used for the treatment of Ewing sarcoma (ES). However, Vin treatment causes sever long-term side effects such as sensory and motor neuropathy, associated with its dose-limiting use. Therefore, there is a need to identify novel strategies to improve the efficacy and reduce toxicities associated with the use of Vin. In this study, we investigated the anti-proliferative effect of combination of tolfenamic acid (TA), a small molecule Sp1 inhibitor, and Vin against ES cell. ES cell lines, CHLA-9 and TC-32, were treated with TA or Vin or TA+Vin combination, and cell viability was assessed at 24 h, 48 h, 72 h post-treatment. Effect of TA or Vin or TA+Vin combination treatment on cell cycle phase distribution and apoptosis was evaluated using propidium iodide cell cycle assay and Annexin V flow cytometry respectively. Apoptosis markers, Caspase 3/7 activity and cleaved-PARP (c-PARP) protein levels were measured. Cell cycle markers (Cyclin A, Cyclin B1) were evaluated using Western blot analysis. Cardiotoxicity was assessed using H9C2 cardiomyocytes. Results revealed that TA and Vin treatment caused time- and dose- dependent inhibition of cell viability. Interestingly, TA+Vin combination treatment significantly inhibited (~ 80%, p value < 0.05) cell viability as compared to TA or Vin alone in ES cells. The inhibition of cell viability was accompanied by inhibition Sp1 and Survivin. TA+Vin combination treatment significantly (p value < 0.05) increased Caspase 3/7 activity which strongly correlated with the increased c-PARP level and Annexin V staining in ES cells. TA and Vin agent alone and combination treatment caused G0/G1 arrest

(TA), G2/M (Vin) or G2/M (TA+Vin) arrest in the ES cells. This G2/M cell cycle arrest was accompanied by upregulation of Cyclin B1 and inhibition of Cyclin A protein expression. TA or Vin or TA+Vin did not cause toxicity, as measured using cell viability assay of H9C2 cardiomyocyte cells. Taken together, results of this study suggest that TA could enhance the anti-proliferative activity of Vin (anti-neoplastic agent), TA+Vin combination could be developed as safe and more effective therapeutic strategy against ES cells.

INTRODUCTION

Ewing sarcoma (ES) is second most common tumor of soft tissue and bone that includes conventional ES and peripheral primitive neuroectodermal tumor (pPNET). It is a highly aggressive and undifferentiated form of neoplasm that usually arises in young adults and children's. Standard care treatment strategies for ES include use of combination chemotherapy treatments along with surgery or radiation therapy (Jemal, Siegel et al. 2010, Jo and Fletcher 2014). Vincristine, an anti-mitotic agent and microtubule destabilizer isolated from leaves of *Catharanthus roseus*, is widely used as an anti-cancer agent for the treatment of cancer. Intensive combination of chemotherapies comprising of Vincristine and other anti-cancer agent such as topotecan, cyclophosphamide, etoposide, ifosfamide, and doxorubicin is often used for the treatment of several pediatric cancers. Vincristine is a common agent in the combination chemotherapeutic regimens used for ES treatment (Ferrari, Palmerini et al. 2010, Kebudi, Cakir et al. 2013, Mascarenhas, Felgenhauer et al. 2016). These strategies have been used for the treatment of pediatric cancers including ES for last five decades. However, these patients suffer from sever neurological and hepatic toxicities associated with their use (Yap, Baker et al. 1980, Paulussen, Ahrens et al. 1998, Simsek, Uner et al. 1998, Thompson, George et al. 1999, Breitfeld, Lyden et al. 2001). Despite the advancements in the treatment modalities, the long term side effects associated with the use of chemotherapy such as secondary malignancies, morbidity issues and relapse after treatment raises a need for innovative treatment modalities for the cure of ES (Bacci, Picci et al. 1998, Craft, Cotterill et al. 1998). Thus, alternative combination treatment strategies are currently under investigation in our lab that aims at identifying a safer and more effective treatment for ES treatment.

Recently, by investigating into the transcription factors and their downstream targets, we have reported that ES cells expressed high levels of Specificity protein 1 (Sp1) and survivin protein levels (Shelake, Sankpal et al. 2016). Sp1, a zinc finger family transcription factor, regulates key cellular processes including but not limited to cell proliferation, survival and apoptosis, and is linked in the pathogenesis of several cancers. Sp1 regulates wide array of genes, especially survivin (an inhibitor of apoptosis protein) that is shown to be associated with the aggressiveness and poor prognosis of multiple cancers. We and others have demonstrated that targeting Sp1 and survivin with a small molecule inhibitor, tolfenamic acid (TA), induces anti-proliferative activity in several adult and pediatric cancers (Kim, Cho et al. 2013, Chang, Kang et al. 2014, Pathi, Li et al. 2014, Sutphin, Connelly et al. 2014, Basha, Connelly et al. 2016, Sankpal, Ingersoll et al. 2016, Sankpal, Nagaraju et al. 2016, Shelake, Sankpal et al. 2016). TA is non-steroidal anti-inflammatory drug (NSAID) approved for the migraine headaches treatment in Europe and Africa (Vaitkus and Pauza 2002). Moreover, we have previously demonstrated that TA induces anti-proliferative activity of anti-neoplastic agents in adult (ovarian, colon) and pediatric cancers (neuroblastoma) (Shelake, Eslin et al. 2015, Sankpal, Ingersoll et al. 2016, Sankpal, Nagaraju et al. 2016).

In this investigation, our aim was to study the efficacy of TA and Vincristine combination treatment against ES cells. We found that TA+Vin combination treatment caused inhibition of cell viability, induced G2/M arrest and increased apoptosis in ES cells than single agent alone. Our results also revealed that TA+Vin combination treatment decreased Sp1 and survivin expression and increased c-PARP levels as well as altered regulatory Cyclin (Cyclin B1, Cyclin A) protein expression.

MATERIAL AND METHODS

Cell lines and cell culture:

Ewing sarcoma (ES) cell lines CHLA-9 and TC-32 were obtained from cell culture repository at Children's Oncology Group (COG), Texas Tech University Health Science Center, Lubbock. Cells were grown in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 4mM L-Glutamine, 1X ITS (5 µg/mL Insulin, 5 µg/mL Transferrin and 5 ng/mL Selenous Acid) and fetal bovine serum. When reached to confluency, cells were passaged using puck's EDTA (140 µM NaCl, 5 µM KCl, 5.5 mM Glucose, 4 mM NaHCO3, 13 µM Phenol Red, 0.8 mM EDTA, and 9 mM HEPES. pH 7.2-7.3). Cells were maintained at 37°C incubator and 5% CO2. H9C2 cells were gift from Dr. Andras Lacko (University of North Texas Health Science Center, Fort Worth, USA), and grown in DMEM cell culture media supplemented with 10 % fetal bovine serum and maintained at 37°C incubator with 5% CO2.

Chemicals and Reagent:

TA, Vincristine, dimethyl sulfoxide (DMSO), and Actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Sp1, Cyclin B1 and Cyclin D1 antibodies was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and Cyclin A and c-PARP antibodies were procured from Cell Signaling (Danvers, MA). Survivin antibody was purchased from R & D Systems (Minneapolis, MN). Dulbecco's phosphate-buffered saline was purchased from Hyclone Laboratories (Logan, Utah). ITS premix was purchased from Corning (Bedford, MA). Cell Titer-Glo kit and Caspase 3/7 assay kit was obtained from Promega (Madison, WI). PE-Annexin V apoptosis assay kit was obtained from BD Bioscience (San Diego, CA). Bicinchoninic acid

(BCA) protein assay kit and super-signal west dura chemiluminiscence kit were purchased from Pierce (Rockford, IL).

Cell Viability Assay:

ES cells were grown in IMDM media and treated with DMSO (control) or TA and Vin or TA+Vin and cell viability was measured using Cell Titer-Glo kit (Promega, Madison, WI). Briefly, 4000 cells per well were plated in 96-well white walled clear bottom plates (Lonza, Basel, Switzerland) and incubated for 24 h at 37°C. CHLA-9 and TC-32 cells were treated with increasing concentration of TA (10-20 µg/ml) or Vin (0-2 ng/ml) for 24 h, 48 h and 72 h. At the end of the incubation period, 100 µl of assay reagent was added, mixed, and the plate was incubated in the dark for 20 min. Luminescence values were obtained from each well using SYNERGY HT microplate reader. All treatments were performed in triplicate and data was normalized for control (DMSO-vehicle treatment) cells and graphed as percent cell viability versus concentration.

Caspase 3/7 Assay:

CHLA-9 and TC-32 cells were treated with TA or Vin or TA+Vin combination and the activity of caspase 3/7 was evaluated using Caspase-Glo 3/7 kit (Promega, Madison, WI), according to manufacturer instructions. Briefly, 4,000 cells/well were plated in a 96-well white walled clear bottom plate (Lonza, Basel, Switzerland) in two sets (cell viability and caspases 3/7). After 24 h, cells were treated with DMSO (control) or TA or Vin, or TA+Vin (optimized drug concentrations). 48 h post-treatment, the assay (CellTiter-Glo or Caspase-Glo 3/7) reagent (100 µl/well) was added, mixed and the plates were incubated in the dark for 60 min. Luminescence

was measured using SYNERGY HT microplate reader. The activity of Caspase 3/7 was normalized with cell viability. All the treatments were performed in triplicate and the data were presented as mean±SD.

Western Immunoblotting:

ES cells were cultured in 100 mm dishes and treated with DMSO (control) or TA and Vin alone or TA+Vin combination. Whole cell lysates were prepared and the expression of proteins of interest was determined using Western blot analysis. Briefly, cells were harvested at 48 h post-treatment, washed twice with ice-cold PBS, and re-suspended in cell lysis buffer containing protease inhibitor. Samples were incubated at 4°C for 30 min, followed by centrifugation at 12,000 rpm for 15 min at 4°C. Protein estimation was performed using BCA protein assay kit. Protein extracts (25 µg of whole cell lysate protein) were boiled with loading buffer containing 2-mercaptoethanol, separated using 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The blots were blocked with blocking buffer containing 5 % (w/v) nonfat dry milk in 10 mmol/L Tris (pH 7.5), 10 mmol/L sodium chloride, and 0.1% Tween 20 (TBST) for 30-60 min at room temperature. Blots were incubated with the primary antibody for overnight at 4°C. The antibody labeled blots were washed three times with TBST for 15 min and incubated with 1:5000 dilution of horse-radish peroxidase conjugated secondary antibody for 2 h. Bands were visualized using Supersignal West Dura using UVP, LLC Bio-Imager.

Annexin V Apoptosis Assay:

Apoptotic cells were measured using PE-Annexin V/7-AAD apoptosis detection kit (BD Biosciences). Briefly, cells were harvested after treatment with vehicle (control) or individual drug (TA or Vin), or combination of investigational agents for 48 h. Cells were incubated with PE-Annexin V antibody and 7-AAD for 15 min in 1X binding buffer and Annexin V+/7-AAD+ cells were analyzed using FC500 flow cytometer. Data was analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR).

Propidium Iodide (PI) Cell Cycle Analysis:

CHLA-9 and TC-32 cells were plated in 6 wells plates. Cells were treated with DMSO (control), TA (15 μg/ml). Vin (0.5 ng/ml) or TA (15 μg/ml) +Vin (0.5 ng/ml) and, processed for cell cycle analysis. After 12 h and 24 h of treated cells were harvested, washed once with PBS and fixed in 1 ml cold 70% ethanol for overnight at 4^0 C. Fixed cells were stained with (0.20 μg/ml PI and 20 μg/ml RNAase A in 1X PBS), and incubated at room temperature for 15 min. Cell cycle phase distribution was studied using FC 500 flow cytometer. Data was analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR) and represented as cell count vs. PI intensity (DNA content).

RESULTS

Effect of TA and Vincristine on cell viability of ES cells

The anti-proliferative activity of TA and Vin was investigated using CHLA-9 and TC-32 cell lines. To obtain the working concentrations for TA and Vin, ES cell lines (CHLA-9 and TC-32) were treated with increasing concentrations of TA (0-20 µg/ml) or Vin (0-2 ng/ml), and cell viability was assayed at 24, 48 and 72 h post-treatment. Results revealed that both TA and Vin caused a dose- and time- dependent inhibition of cell viability in ES cell lines (Figure 4.1). The derived IC_{50} values for TA for these cell lines ranged between 12-15 µg/ml, and that of Vin ranged between 0.35-0.63 ng/ml at 48 h post-treatment. Our results indicate that ES cells are sensitive to clinical/therapeutic concentrations of TA and Vin. After analyzing the data, 15 µg/ml of TA and 0.5 ng/ml of Vin were chosen for the combination treatment.

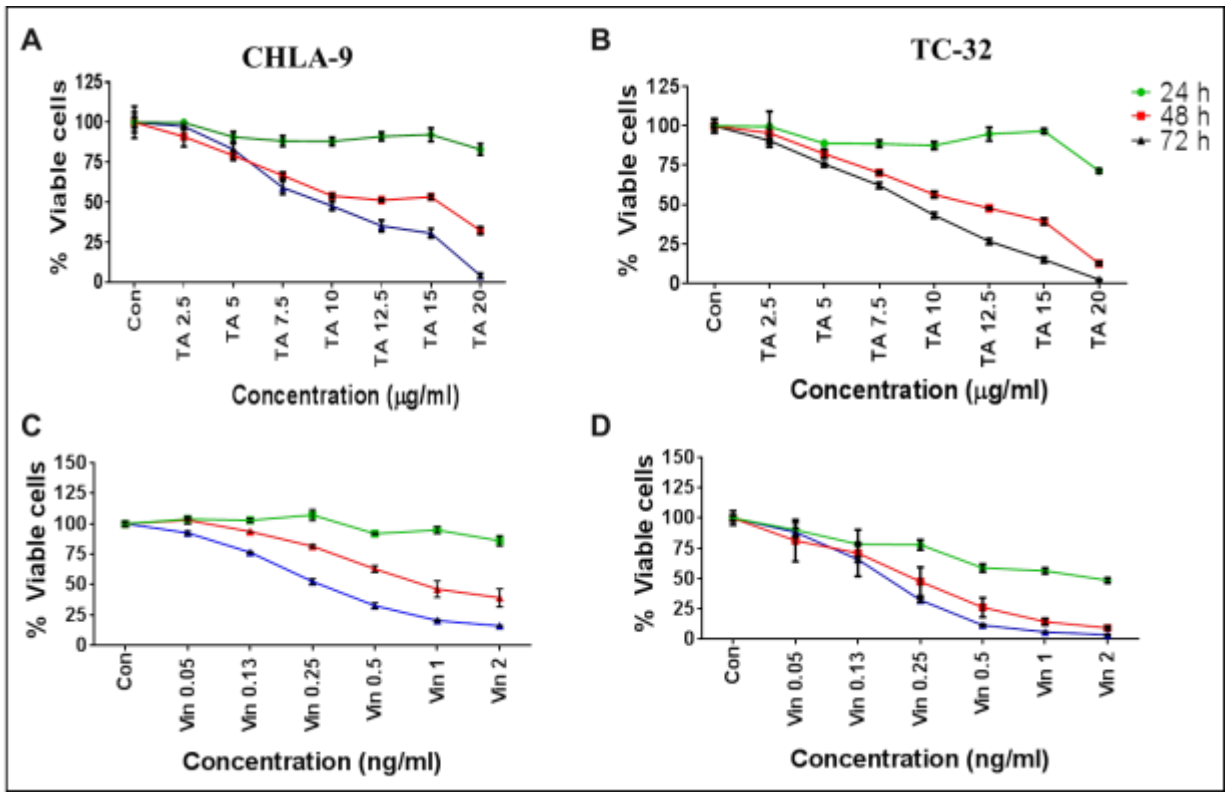
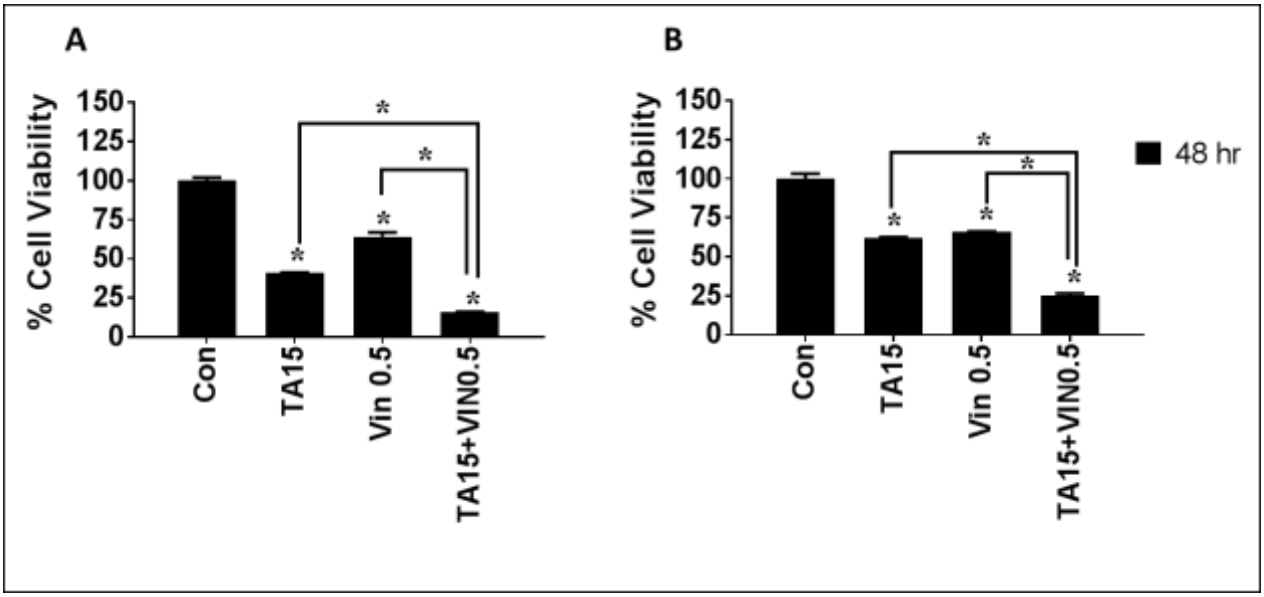


Figure 4.1: **TA and Vin decreases cell viability of ES cells in a dose- and time-dependent manner.** Human ES cells, CHLA-9 (A and C), TC-32 and (B and D) were treated with DMSO (Control) or increasing concentrations (0-20 μg/ml) of TA and (0-5 ng/ml) of Vin, and the cell viability assay was performed using CellTiter-Glo kit (Promega) at 24 h, 48 h and 72 h post-treatment. Data shown indicate the percent cell viability normalized to control (DMSO). Bars represent the mean ± SD of triplicate determinations.

Effect of TA+Vin combination treatment on Sp1 and survivin expression

The effect of TA and Vincristine alone or TA+Vin combination treatment on cell viability of ES cells assessed using CellTiter-Glo assay. Sp1 and survivin expression was determined using Western immunoblot analysis. CHAL-9 and TC-32 cells were treated with optimized doses of TA (15 μg/ml) and Vin (0.5 ng/ml) or TA+Vin combination. The results showed that TA+Vin combination treatment significantly inhibited cell viability of ES cells compared to the TA or Vin alone. Interestingly, the inhibition of cell viability was accompanied by decreased Sp1 and survivin protein expression at 48 h post-treatment (Figure 4.2).



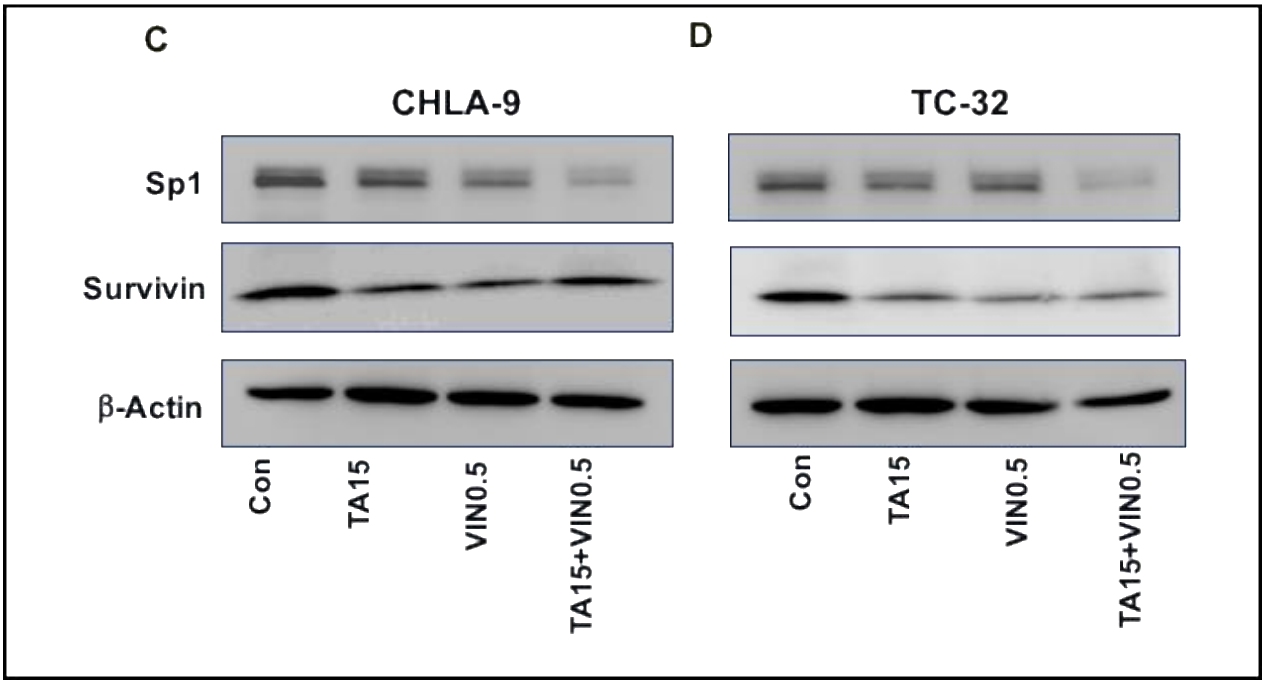


Figure 4.2: Combination treatment of TA and Vin inhibits Sp1 and survivin protein expression. CHLA-9 (A, C), TC-32 (B, D) cells were treated with DMSO, TA (15 μg/ml), Vin (0.5 ng/ml) or combination of TA + Vin. 48 h post-treatment cell viability was measured using CellTiter-Glo kit (Promega). Data shown indicate the percent cell viability normalized to control

(DMSO). Bars represent the mean of triplicate determinations ± SD. Whole cell lysates were prepared at 48 h post-treatment and the expression of Sp1 and Survivin was evaluated by Western blot analysis. β−actin was used as loading control. Representative gels are shown in the figure.

Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0 (* represents p value < 0.05).

CON: Control (DMSO); TA 15: Tolfenamic acid 15 μg/ml; Vin0.5: Vincristine 0.5 ng/ml. TA15+Vin 0.5: Tolfenamic acid 15 μg/ml + Vincristine 0.5 ng/ml.

Effect of TA and Vincristine on apoptosis in ES cells

ES cells, CHLA-9 and TC-32 were treated with TA and Vin alone or in combination. The effect of TA and Vin was studied by performing Annexin V flow cytometry and apoptosis markers (caspase 3/7 activation and c-PARP protein level) were measured. 48 h post-treatment TA+Vin combination treatment significantly increased the Annexin V positive cells in CHLA-9 (1.6 fold) and TC-32 (4.6 fold) compared to single agent alone (Figure 4.3).

The increased apoptosis strongly correlated with increased Caspase 3/7 level as well as c-PARP protein expression (Figure 4.4). These results indicate that TA enhances the anti-proliferative effect of Vin as observed by increased Annexin V staining and increased apoptosis markers (Caspase 3/7 activity and c-PARP protein levels) in TA+Vin combination treatment.

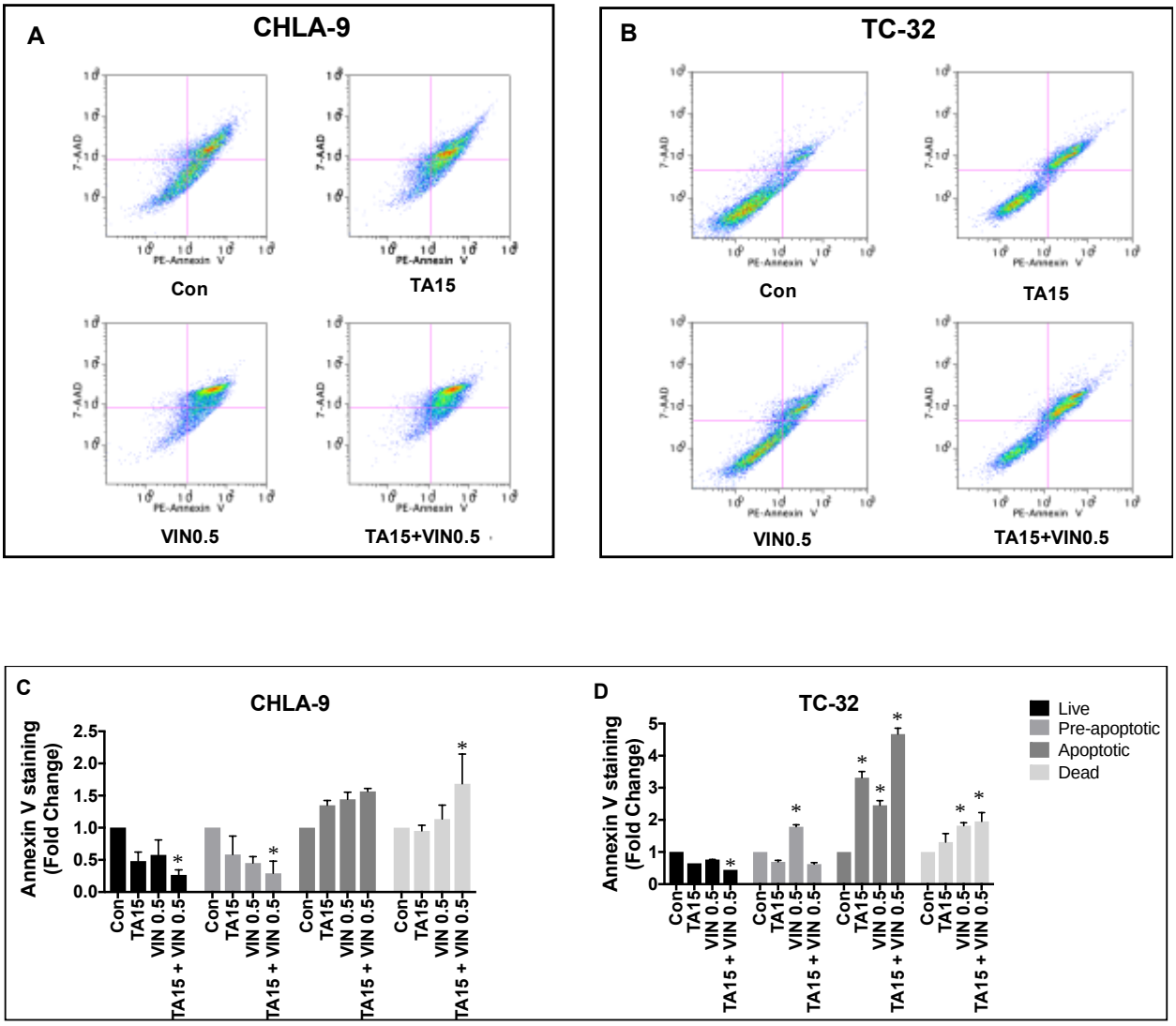


Figure 4.3: Combination treatment with TA and Vin induces apoptosis. CHLA-9 (A), TC-32 (C) cells were treated with DMSO, cells were treated with DMSO, TA (15 μg/ml), Vin (0.5 ng/ml) or TA + Vin combination. 48 h post-treatment, apoptosis was analyzed using Annexin V-PE/7-AAD kit on BD LSR II (BD Bioscience). Statistical graph of Annexin V+/ 7-AAD+ positive apoptotic cells in CHLA-9 (C) and TC-32 (D) is shown. The graphs represent mean±SD of results obtained from three independent experiments. Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0 (* represents p value < 0.05).

CON: Control (DMSO); TA 15: Tolfenamic acid 15 µg/ml; Vin0.5: Vincristine 0.5 ng/ml. TA15+Vin 0.5: Tolfenamic acid 15 µg/ml + Vincristine 0.5 ng/ml.

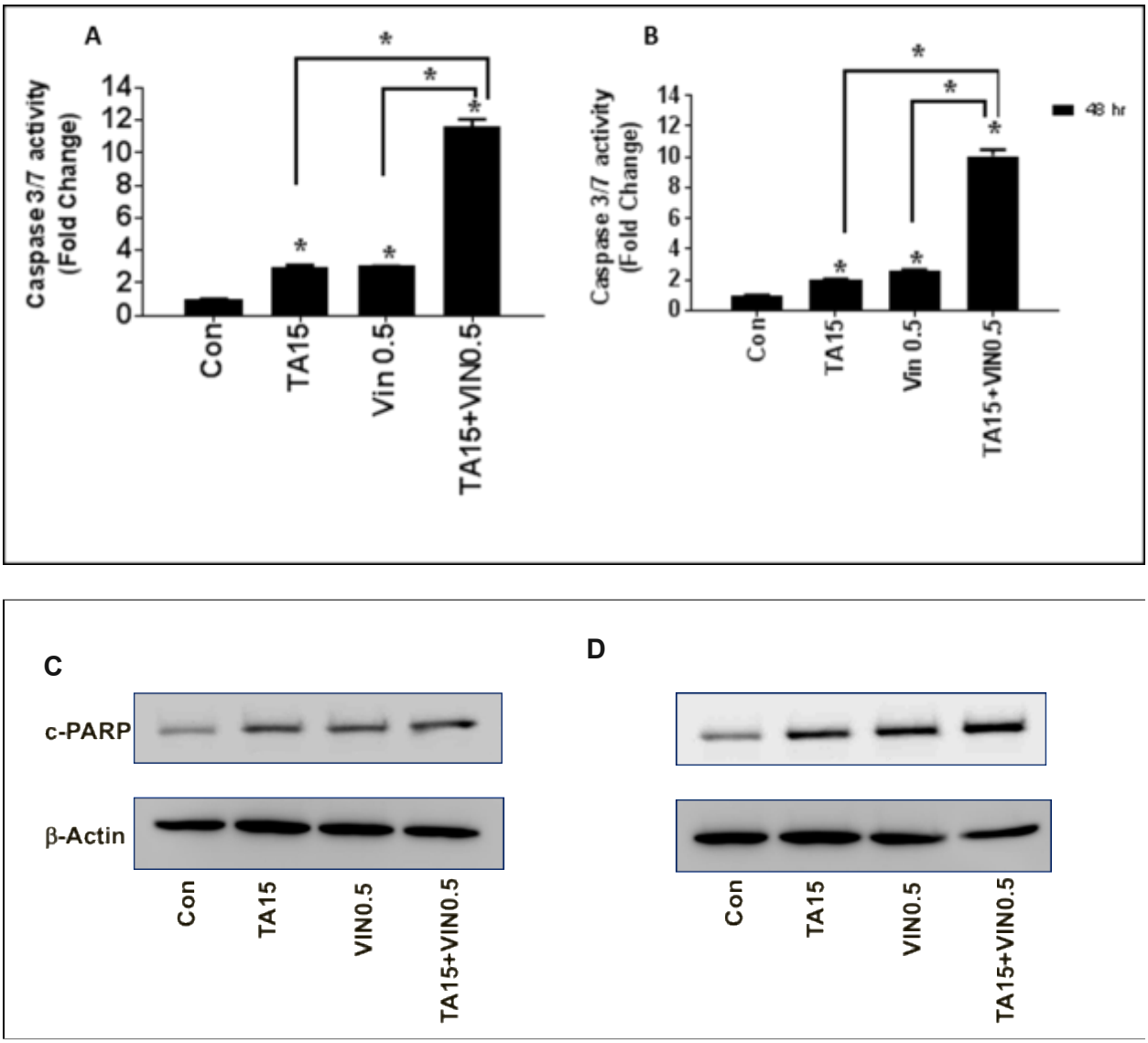


Figure 4.4: Co-treatment with TA and Vin significantly up-regulated caspase-3/7 activity and c-PARP expression. CHLA-9 (A, C), TC-32 (B, D) cells were treated with DMSO, TA (15 µg/ml), Vin (0.5 ng/ml) or combinations of TA + Vin. 48 h post-treatment, Caspase 3/7 activity was measured using caspase 3/7 Glo kit. Whole cell lysates were prepared and c-PARP protein expression was evaluated by Western blot analysis at 48 h post-treatment. The data were

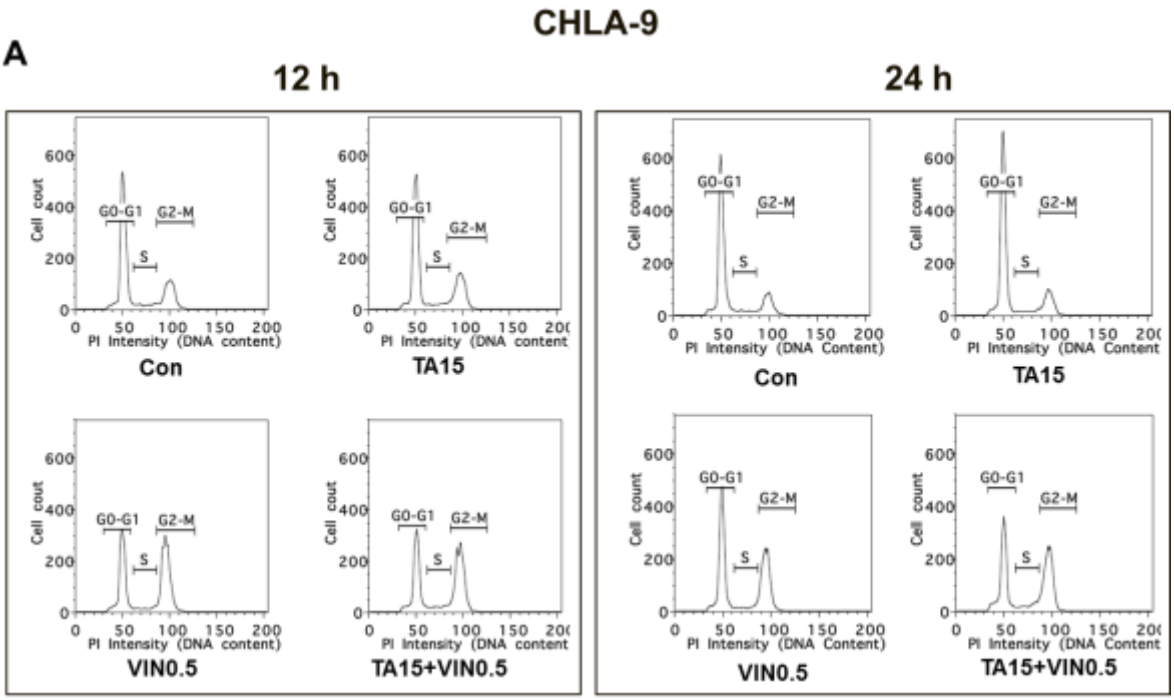
obtained from three independent experiments. Representative Western blot images are shown in the figure. All data represent the mean ± SD of three independent determinations. Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0. (* represents *p* value < 0.05).

CON: Control (DMSO); TA 15: Tolfenamic acid 15 μg/ml; Vin0.5: Vincristine 0.5 ng/ml. TA15+Vin 0.5: Tolfenamic acid 15 μg/ml + Vincristine 0.5 ng/ml.

Effect of TA and Vincristine on cell cycle phase distribution

The combination treatment of TA + Vin dramatically decreased cell proliferation in ES cells. Since this effect could be accompanied by either induction of cell cycle arrest or increased apoptosis or combination of both. In order to determine the involvement of alterations in cell cycle distribution, we performed propidium iodide flow cytometric assay to evaluate the effect of TA and Vin alone or TA+Vin combination treatment on cell cycle. TA has been shown to induce G0/G1 cell cycle arrest in neuroblastoma cells (Eslin, Sankpal et al. 2013). Consistent with previous results, TA induced G0/G1 cell cycle arrest in ES cells. The mechanism of action of Vincristine includes destabilization of microtubule formation followed by G2/M arrest. Vin treatment significantly increased G2/M arrest in CHLA-9 cells (Con: 45.5%± 3.8; TA+Vin: 48.7 ± 2.7) and TC-32 cells (Con: 26.8%± 0.7; TA+Vin: 49.3± 2.7) as early as 12 h after treatment and sustained through 24 h (Figure 4.5).

A



B

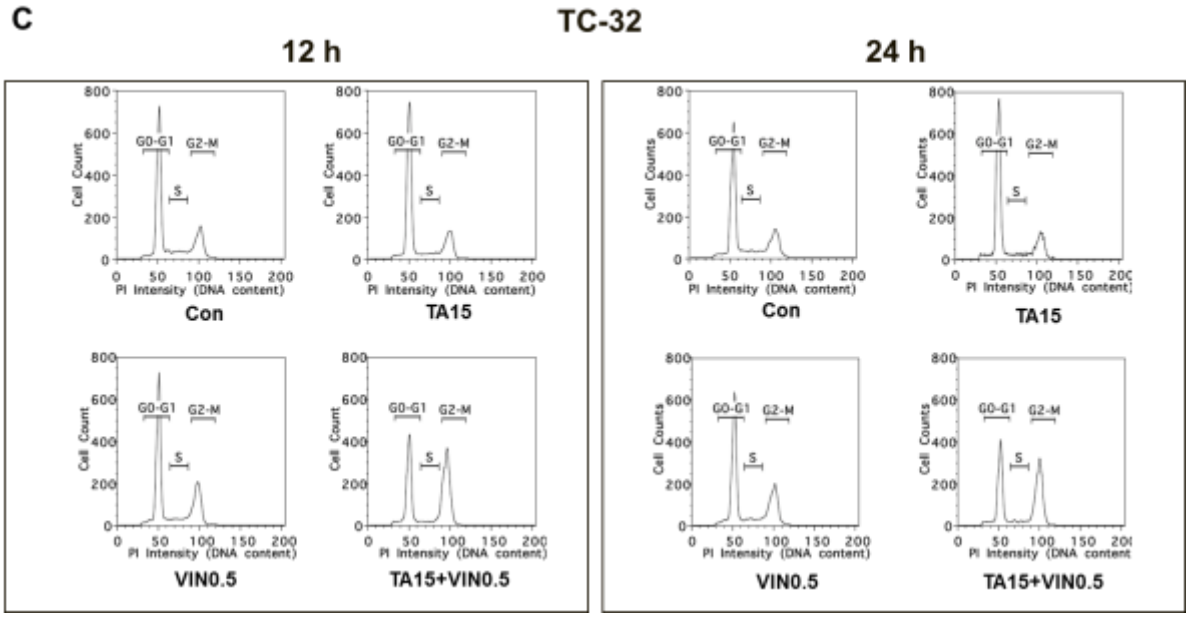
CHLA-9 (12 h)

	Con	TA15	VIN 0.5	TA15+VIN0.5
G0/G1	66.6±0.3	64.3±1.8	43.5±1.3	44.5±2.0
S	8.5±1.2	8.2±0.9	10.3±2.1	6.4±0.8
G2/M	24.5±1.6	27.2±2.7	45.5±3.8*	48.8±2.7*

CHLA-9 (24 h)

	Con	TA15	VIN 0.5	TA15+VIN0.5
G0/G1	71.8±1.1	74.6±0.6	53.1±0.7	46.1±2.0
S	8.1±0.9	6.2±0.3	6.0±0.6	7.1±1.3
G2/M	19.9±0.3	18.9±0.9	40.5±1.4*	46.2±1.2*

C



D

TC-32 (12 h)

	Con	TA15	VIN 0.5	TA15+VIN0.5
G0/G1	61.3±1.5	64.9±3.9	57.7±1.2	40.6±2.4
S	14.7±2.6	9.6±1.2	12.8±2.3	7.3±0.9
G2/M	19.5±3.0	23.7±3.8	26.8±0.7*	49.3±2.7*

TC-32 (24 h)

	Con	TA15	VIN 0.5	TA15+VIN0.5
G0/G1	60.4±1.8	68.2±2.6	55.3±4.6	40.7±2.9
S	11.4±1.0	8.4±0.8	9.1±0.8	6.9±0.3
G2/M	24.3±4.3	21.6±2.7	33.±5.7*	50.7±3.5*

Figure 4.5: Co-treatment with TA and Vin significantly up-regulated caspase-3/7 activity and c-PARP expression. CHLA-9 (A, C), TC-32 (B, D) cells were treated with DMSO, TA (15 μg/ml), Vin (0.5 ng/ml) or combinations of TA + Vin. Cell cycle phase distribution was assayed at 12 h and 24 h post-treatments. The percent distribution of cells in G0/G1, S, and G2/M phase following single agent or combination treatment is shown in representative cell cycle histograms and values are listed in table. All data represent the mean±SD of the results obtained from three independent experiments. Statistical analysis performed by two-way ANOVA using GraphPad Prism V6.0 (* represents *p* value < 0.05).

CON: Control (DMSO); TA 15: Tolfenamic acid 15 μg/ml; Vin0.5: Vincristine 0.5 ng/ml. TA15+Vin 0.5: Tolfenamic acid 15 μg/ml + Vincristine 0.5 ng/ml.

In order to delineate the signaling pathway involved in effectiveness of TA+Vin, we measured G2/M cell cycle markers (Cyclin A and Cyclin B1). Results revealed that TA+Vin treatment resulted inhibition of upregulated Cyclin B1 which was accompanied by decrease in Cyclin A protein expression than control (Figure 4.6).

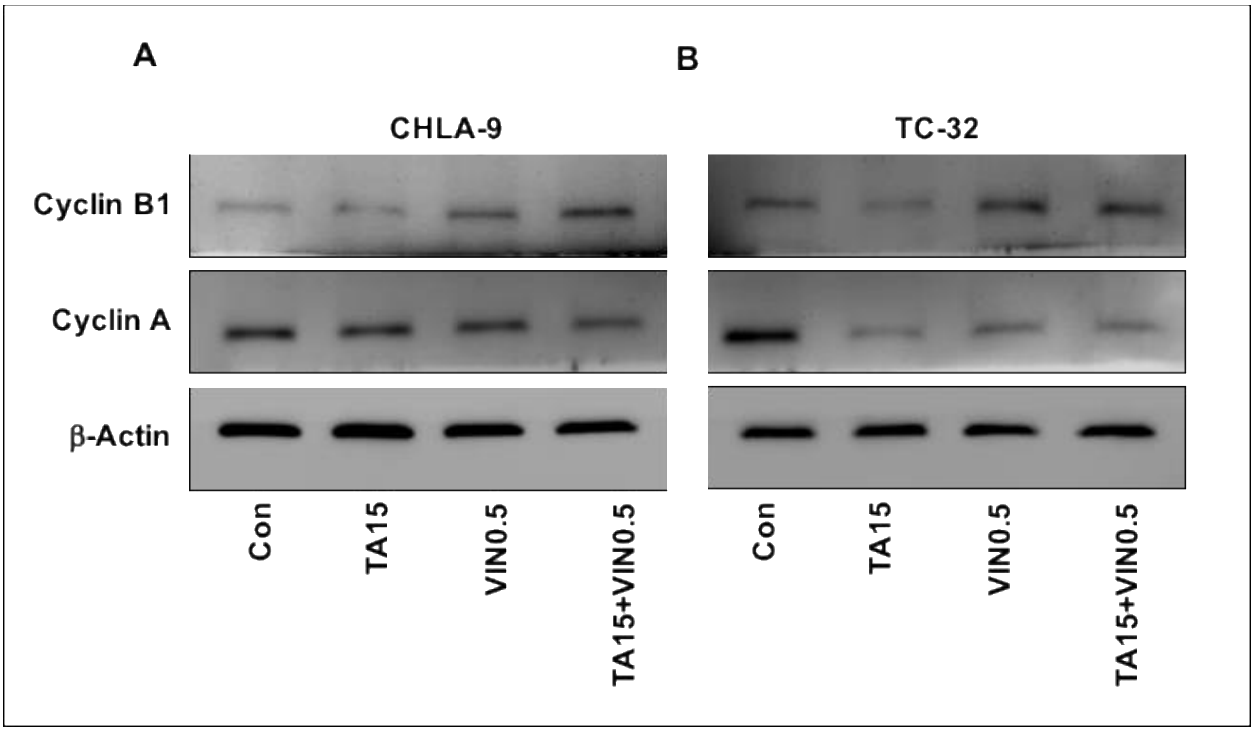


Figure 4.6: Combination treatment of TA and Vin alters G2/M cell cycle regulatory protein expression. CHLA-9 (A), TC-32 (B) cells were treated with DMSO, TA (15 μg/ml), Vin (0.5 ng/ml) or combination of TA + Vin. Whole cell lysates were prepared at 48 h post-treatment and the protein expressions of Cyclin A and Cyclin B1 were evaluated by Western blot analysis. β-actin was used as loading control. Representative gel images are shown in the figure.

CON: Control (DMSO); TA 15: Tolfenamic acid 15 μg/ml; Vin0.5: Vincristine 0.5 ng/ml. TA15+Vin 0.5: Tolfenamic acid 15 μg/ml + Vincristine 0.5 ng/ml.

Effect of TA and Vin on cell viability OF H9C2 cells.

The use of NSAIDs is linked to the risk of cardiotoxicities in healthy individuals as well as in patients suffering from cardiovascular disorders. Since TA is a NSAID drug, we evaluated the effect of TA and Vin single agent or TA+Vin combination treatment on H9C2 cardiomyocyte cells. H9C2 cells were treated with DMSO (vehicle), TA 10 ug/ml, TA 15 ug/ml, Vin 0.5 ng/ml alone or combination of TA+Vin, and cell viability was measured at 24 h, 48 h and 72 h post-

treatments. Results showed that TA and Vin alone or TA+Vin combination treatment does not induce toxicity in H9C2 cells (Figure 4.7).

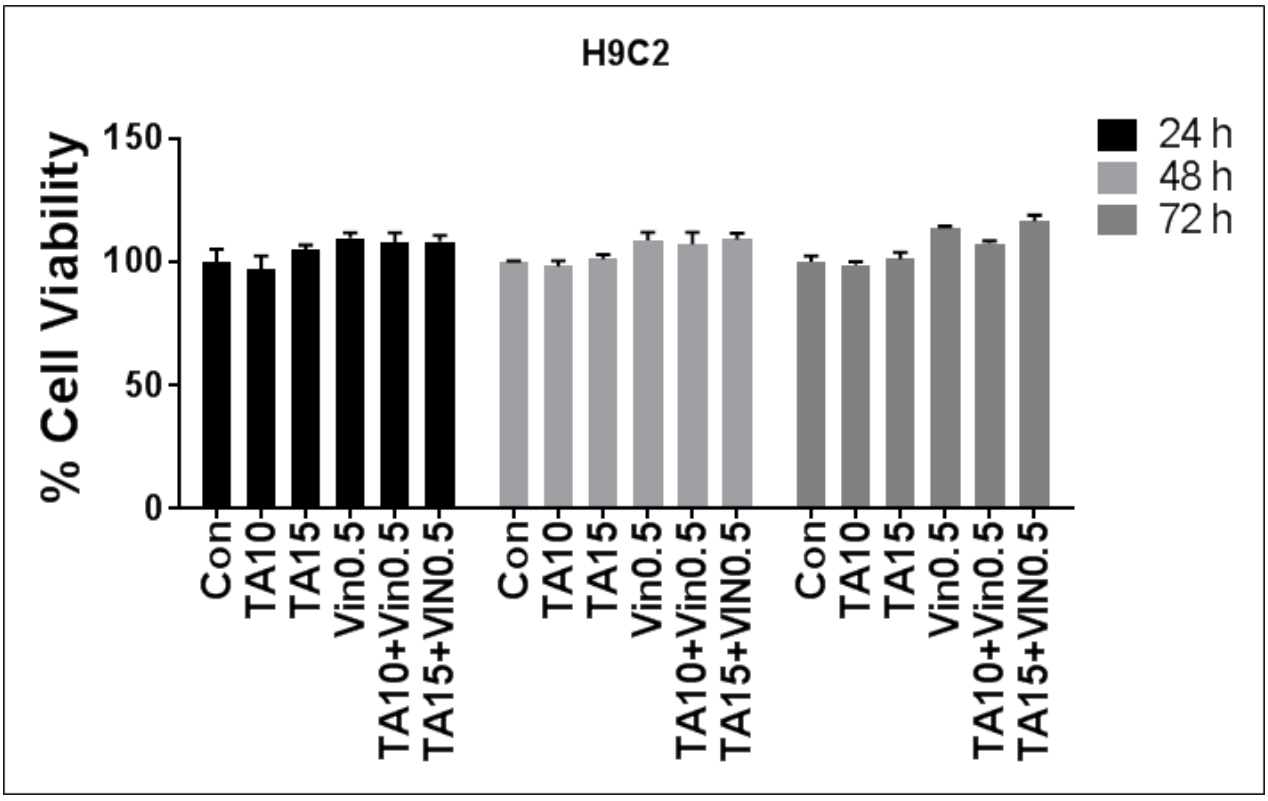


Figure 4.7: TA and Vin does not cause toxicity in H9C2 cardiomyocyte cells. H9C2 cells were treated with DMSO (Control) or TA and (10 or 15 μg/ml) or Vin (0.5 ng/ml), or TA+Vin (indicates doses). Cell viability assay was performed using CellTiter-Glo kit (Promega) at 24 h, 48 h and 72 h post-treatment. Data shown indicate the percent cell viability normalized to control (DMSO). Bars represent the mean of triplicate determinations ± SD.

CON: Control (DMSO); TA 10: Tolfenamic acid 10 μg/ml; TA 15: Tolfenamic acid 15 μg/ml; Vin0.5: Vincristine 0.5 ng/ml; TA10+Vin 0.5: Tolfenamic acid 10 μg/ml + Vincristine 0.5 ng/ml; TA15+Vin 0.5: Tolfenamic acid 15 μg/ml + Vincristine 0.5 ng/ml.

DISCUSSION

Ewing sarcoma is the second most common tumor of bone and soft tissue that affects adolescent and young adults. Vincristine, a Vina alkaloid, is an anti-neoplastic agent that is currently used a component of several combination treatment regimens approved to treat several adult and pediatric malignancies along with ES. However, its applications are limited due to a peripheral neuropathy associated with its use. This Vincristine-induced peripheral neuropathy (VIPN), although undetectable in early stages, severely affects the quality of long-term survivors (Grisold, Cavaletti et al. 2012, Lavoie Smith, Li et al. 2015, Mora, Smith et al. 2016). Despite the success and advancements in the treatment modalities, very little progress has been made to reduce the toxicities such as VIPN and improve the effectiveness of anti-neoplastic agents. In this study, we investigated the ability of a non-steroidal anti-inflammatory molecule, tolfenamic acid, to induce anti-proliferative activity of Vin in ES cells. Both TA and Vin treatment inhibited the ES cell viability in time-dose-dependent manner and that this inhibition was greatly enhanced when two agents were used in combination (Figure 4.1).

Our lab and others have previously showed TA induces anti-proliferative activity in several types of cancer cells by inhibiting Sp1 and survivin protein expression including ES cells (Eslin, Lee et al. 2013, Eslin, Sankpal et al. 2013, Sankpal, Lee et al. 2013, Basha, Connelly et al. 2016, Shelake, Sankpal et al. 2016). Consistent with the previously published literature, we showed that TA inhibits Sp1 and survivin in ES cells and that TA+Vin combination treatment further enhanced the Sp1 and survivin inhibition. Although there are no previous reports of the modulation of Sp1 in response to Vincristine treatment, our results revealed that Vin treatment

causes a very moderate downregulation of Sp1 in CHLA-9 cells but not in TC-32 cells (Figure 4.2).

Survivin, an inhibitor of apoptosis protein has been shown to be upregulated in several cancers including sarcomas (Kappler, Kotzsch et al. 2003). Previous reports suggest that Sp1 transcription factor regulate survivin gene expression by modulating its promoter activity (Xu, Zhang et al. 2007). Results of this investigation indicated that TA+Vin combination treatment inhibited both Sp1 and survivin protein expression (Figure 4.2). Our laboratory and others have demonstrated that TA induces proteasome mediated degradation and alters the DNA binding activity of Sp1 transcription factor in cancer cells (Abdelrahim, Baker et al. 2006, Shelake, Sankpal et al. 2016). Most importantly, we have showed that anti-proliferative activity TA could be attributed to its ability to inhibit survivin protein expression as observed in several cancer types (Basha, Ingersoll et al. 2011, Eslin, Sankpal et al. 2013, Sutphin, Connelly et al. 2014, Basha, Connelly et al. 2016, Shelake, Sankpal et al. 2016). Although survivin is known to regulate G2/M cell cycle phase transition (Li, Ambrosini et al. 1998), the effect of Vin, which causes G2/M cell cycle arrest, on survivin protein expression is yet unclear. Previously, Vin treatment shown to upregulate survivin protein expression in chromic myeloid leukemia cells (Souza, Vasconcelos et al. 2011). However, recent report suggests that Vin enhanced the anti-cancer activity of Cotylenin A partially by inhibiting expression of anti-apoptotic proteins such as cIAP, and Survivin (Takahashi, Honma et al. 2015). Therefore, the effect of Vin on survivin expression could depend upon the underlying cellular and genetic context and needs to be further studied in details.

TA or Vin or TA+Vin combination treatment induced anti-proliferative activity in ES cells could be partly due to activation of apoptosis pathways. Annexin V flow cytometry results showed that TA+Vin combination treatment significantly increased apoptotic cells compared to control and TA or Vin single agent (Figure 4.3). Further studies were performed to understand the underlying molecular mechanism involved in the apoptosis process. Caspases, proteins that cleaves regulatory proteins such as PARP, are critical mediators of programmed cell death. Caspase 3/7 are effector caspases that gets activated in both extrinsic (independent on cytochrome C release from mitochondria) and intrinsic apoptosis (dependent on cytochrome C release from mitochondria) pathways (Zou, Henzel et al. 1997, Porter and Janicke 1999, Springer, Azbill et al. 1999). Vincristine and cisplatin, a microtubule disrupting drugs, shown to activate induce Caspase 3/7 activity in ovarian cancer cells via activation of Caspase 8 by unknown mechanism. Moreover, they do not cause any effect on the mitochondrial mediated apoptosis mechanism (Milner, Palmer et al. 2002). We have previously showed that TA induces apoptosis in human neuroblastoma cells via activation of both Caspase 3/7 and Caspase 8/9 (Shelake, Eslin et al. 2015). Several anti-cancer agents have been reported induce apoptosis in cancer cells by inducing Caspase 3/7 activity (Okun, Balakin et al. 2008). In this study, our results revealed that TA+Vin combination treatment significantly increased Caspase 3/7 activity and c-PARP protein expression that strongly correlated with Annexin V flow cytometry results (Figure 4.4.) Therefore, it is possible that TA and Vin combination treatment could synergistically induce activating caspases (Caspase 8 and Caspase 9) and thereby resulting dramatic increase in effector caspase, Caspase 3/7, activation in ES cells.

As pointed out earlier, anti-proliferative activity of TA+Vin combination treatment could involve activation of both apoptosis as well as cell cycle arrest. TA has been shown to induce G0/G1 cell cycle arrest in neuroblastoma cells (Eslin, Sankpal et al. 2013), whereas, Vin has been reported in induce G2/M arrest in several cancer types (Wang, Jin et al. 2003). In this investigation, we assessed the effect of TA and Vin alone or TA+Vin combination treatment on cell cycle phase distribution in ES cells at 12 h and 24 h. TA induces G0/G1 at 24 h and 48 h in ES cells (unpublished data). Our results revealed that TA+Vin combination treatment caused G2/M arrest in ES cells at 12 and 24 h (Figure 4.5). Given the differences in the mechanism of action, it possible that Vin treatment induced cell cycle arrest could precede of TA, and could predominate in TA+Vin combination treatment. The effect of TA+Vin could be attributed to their effect on Cyclins such as (Cylin D1/D2/D3 for G0/G1 and Cyclin A/Cyclin B1 for G2/M phase transition). It has been reported that Vin induces anti-cancer activity in by differentially regulating Cyclin B1 expression in cancer dependent manner. Such as Vin is shown to induce expression of Cyclin B1 in human neuroblastoma and leukemia cancer cells (Tu, Cheng et al. 2013, HuangFu, Chao et al. 2017). Consistent with previous reports and cell cycle phase distribution observations, our results suggest that TA+Vin combination treatment increases Cyclin B1 expression than control in ES cells. It is known that accumulation of Cyclin B1 in cells activates the proteasome mediated degradation of Cyclin A during G2/M cell cycle progression (Henglein, Chenivesse et al. 1994, Yam, Fung et al. 2002). Consistent with this theory, we observed that TA+Vin induced Cyclin B1 strongly correlated with decreased Cyclin A protein expression (Figure 4.6).

Using pre-clinical mouse model, we and others have showed that TA is safe to use and did not cause any apparent toxicity in vital organs (Eskerod 1994, Sankpal, Lee et al. 2013). Non-steroidal anti-inflammatory drugs are reported to cause cardiotoxicity. In this study, we assessed the effect of TA alone or TA+Vin combination treatment on cell viability of H9C2 cardiomyocyte cells. Our results indicate that TA alone or TA+Vin does not cause any toxicity in cardiomyocyte (Figure 4.7). TA is approved by European Medical Association for the treatment of migraine headaches in human. Our laboratory has shown that TA enhances anti-proliferative activity of cis-retinoic acid in neuroblastoma and curcumin in colon cancer cells (Shelake, Eslin et al. 2015, Sankpal, Nagaraju et al. 2016). Thus, further pre-clinical studies are warranted to ascertain use of TA in combination with anti-neoplastic agent for treatment of advanced and metastatic cancer types.

In a summary, this study shows that small molecule NSAID, TA, enhances anti-proliferative response of Vin in ES cells by inhibiting Sp1 and survivin expression. The TA+Vin combination treatment caused significant activation of Caspase 3/7, increased c-PARP expression and induced apoptosis. Among two ES cells, TC-32 cells had higher sensitivity for TA+Vin combination treatment. TA+Vin caused higher anti-proliferative response in TC-32 cells as seen in Annexin V apoptosis and cell cycle analysis than CHLA-9 cells. Vin alone or TA+Vin treatment cause G2/M arrest in both ES cells and was accompanied by increase in Cyclin B1 protein expression. Importantly, TA or Vin or TA+Vin did not cause any changes in H9C2 cardiomyocyte cells supporting safety. Taken together, this result from our investigation provides strong rationale for the use of TA+Vin combination treatment for the ES treatment.

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CHAPTER V

SUMMARY

Specificity protein1 (Sp1) is a zinc-finger transcription factor that belongs to kruppel-life family (KLF) of transcription factors. Sp1 is overexpressed and linked to the aggressiveness and poor prognosis of multiple cancer types (Safe and Abdelrahim 2005, Colon, Basha et al. 2011, Jiang, Cui et al. 2012, Malek, Nunez et al. 2012, Pathi, Li et al. 2014). Although there is strong association of Sp1 in several cancers, strategies targeting Sp1 for clinical application are lacking. Sp1 transcription factor, in addition to being a pro-oncogenic factor, regulates critical genes (eg., cyclin D1, c-fos, c-Met and E2F) important in the promotion of cell growth, (Dong, Wang et al. 1999, Li and Altieri 1999, Wang, Dong et al. 1999, Castro-Rivera, Samudio et al. 2001, Li, Xie et al. 2006), cell survival (eg., survivin, Bcl-2 and Bcl-XL) and angiogenesis promoting growth mediators, their ligands and receptors (VEGF and VEGF receptors) (Abdelrahim, Smith et al. 2004, Jesmin, Sakuma et al. 2004, Stachurska, Kozakowska et al. 2011, Deacon, Onion et al. 2012).

Survivin (a member of IAP family), has been found to be upregulated in several cancers types such as cancers of lung, liver, colorectal (Song, Jung et al. 2009), soft-tissue sarcoma, gastric adenocarcinoma, merkel cell carcinoma, and is shown to be associated with their poor prognosis (Kappler, Kotzsch et al. 2003, Mita, Mita et al. 2008, Knight, Oprea-Ilies et al. 2011, Greve, Sheikh-Mounessi et al. 2012, Hingorani, Dickman et al. 2013, Beishline and Azizkhan-Clifford 2015, Habib, Akhtar et al. 2015, Hu, Hu et al. 2016, Khan, Khan et al. 2016, Liu, Ji et al. 2016, Liu, Huang et al. 2016). In healthy cells, survivin plays a key role in the maintenance of cellular homeostasis that regulates cell division, turnover of caspase and suppresses apoptosis. However, it

prevents apoptosis by binding to effector caspases, Caspase 3/7, thereby inhibiting their activity in uncontrollably proliferating cancer cells (Sun, Cai et al. 1999, Shin, Sung et al. 2001, Rubio, Garcia-Segura et al. 2012, Ding, Li et al. 2010, Xu, Wang et al. 2010, Vranic 2013, Lebelt, Rutkowski et al. 2016). The strategies targeting survivin to induce growth attrition of cancer cell are showing great promises as reported in several *in-vitro* and pre-clinical studies (de Jong, van Oosterwijk et al. 2016, Ferrario, Luna et al. 2016, Qi, Dong et al. 2016, Sam, Sam et al. 2016, Yenkejeh, Sam et al. 2016).

TA is a NSAID used as an anti-inflammatory drug and analgesic in veterinary medicine and treatment of migraines in humans, and is shown to be relatively safe. TA is demonstrated to induce anti-cancer activity by inhibiting Sp family proteins (Sp1, Sp3, and Sp4) and survivin in several adult and pediatric cancers (Basha, Ingersoll et al. 2011, Sankpal, Abdelrahim et al. 2012, Eslin, Lee et al. 2013, Eslin, Sankpal et al. 2013, Sutphin, Connelly et al. 2014). TA-mediated inhibition of Sp1 protein expression is attributed to proteasome-mediated degradation of Sp1 in pancreatic cancer cells (Abdelrahim, Baker et al. 2006). In the oncology care, novel diagnostics and treatment options such as combination of antineoplastic agents, surgery and/or radiation therapy are employed as a standard treatment strategy to improve the effectiveness and lower side effects associated with anti-cancer drugs. However, the treatment of patients suffering from advanced and metastatic cancer remains elusive as these patients often suffer from relapse and more aggressive disease. Although 5-year overall survival of the ES patients ranges between 65-70%, it is 50% less for the patients with metastatic and advanced disease (Gaspar, Hawkins et al. 2015). The research conducted for this dissertation elucidates the efficacy of novel combination treatment strategies comprising of a less toxic NSAID (TA) and anti-neoplastic agent(s) used in the standard care for inducing cell growth inhibition in HRNB and ES cell lines. The results of

this research provide a rationale for designing treatment strategies involving Sp1 inhibitor (for example, TA) along with anti-neoplastic agents. The major findings of this dissertation work are: I) TA enhances the anti-proliferative activity of RA in HRNB cells by inhibiting Sp1 and survivin protein expression. II) TA inhibits cell growth and induces apoptosis in ES cells by inhibiting Sp1 and survivin expression. III) TA induces the anti-cancer activity of vincristine in ES cells by upregulating apoptotic markers and causing G2/M cell arrest.

FUTURE DIRECTIONS:

Although Sp1 and survivin are implicated in the development and progression of several cancers and are considered as druggable targets, studies aiming to target Sp1 and survivin for therapeutic purpose are still lacking. Our expectation is that, such strategies will eventually be tested in clinical setting to introduce effective therapies for the patients. The research presented in this dissertation provides the preliminary evidence to target Sp1 and survivin using TA for inducing anti-proliferative activity in cancer cells. Since these studies are conducted using *in vitro* assays, it is essential to confirm our results using *in vivo* assays for translational applications. Studies using patient-derived cells are highly recommended for assessing the response of the proposed combination treatments. One of the primary reasons for poor prognosis in relapsed cancer patients is resistance to chemotherapy. Since there is ample evidence for the association of Sp1 and survivin in drug resistance, studies may be conducted to determine the effect of TA against chemotherapy resistance by evaluating the markers for drug resistance and/or using resistance cell lines.

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