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Clotam enhances anti-proliferative effect of vincristine in Ewing sarcoma cells

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Abstract

Current therapeutic strategies used in Ewing sarcoma (ES) especially for relapsed patients have resulted in modest improvements in survival over the past 20 years. Combination therapeutic approach presents as an alternative to overcoming drug resistance in metastatic ES. This study evaluated the effect of Clotam (tolfenamic acid or TA), a small molecule and inhibitor of Specificity protein1 (Sp1) and survivin for sensitizing ES cell lines to chemotherapeutic agent, vincristine (VCR). ES cells (CHLA-9 and TC-32) were treated with TA or VCR or TA + VCR (combination), and cell viability was assessed after 24/48/72 h. Effect of TA or VCR or TA + VCR treatment on cell cycle arrest and apoptosis were evaluated using propidium iodide, cell cycle assay and Annexin V flow cytometry respectively. The apoptosis markers, caspase 3/7 (activity levels) and cleaved-PARP (protein expression) were measured. Cardiomyocytes, H9C2 were used as non-malignant cells. While, all treatments caused time- and dose-dependent inhibition of cell viability, interestingly, combination treatment caused significantly higher response (~80% inhibition, $p < 0.05$). Cell viability inhibition was accompanied by inhibition of Sp1 and Survivin. TA + VCR treatment significantly ($p < 0.05$) increased caspase 3/7 activity which strongly correlated with upregulated c-PARP level and Annexin V staining. Cell cycle arrest was observed at G0/G1 (TA) or G2/M (VCR and TA + VCR). All treatments did not cause cytotoxicity in H9C2 cells. These results suggest that TA could enhance the anti-cancer activity of VCR in ES cells. Therefore, TA + VCR combination could be further tested to develop as safe/effective therapeutic strategy for treating ES.

Keywords Ewing sarcoma · Tolfenamic acid · Vincristine · Sp1 · Survivin

Introduction

Ewing sarcoma (ES) is the second most common soft tissue and bone tumor type and is characterized by a unique chromosomally rearranged fusion protein EWS/FLI1. It is a highly aggressive and undifferentiated form of neoplasm that usually arises in young adults and children. Standard care treatment strategies for ES includes use of combination chemotherapy treatments along with surgery or radiation therapy [1, 2].

Intensive combination of chemotherapy agents comprising of Vincristine (VCR) and other anti-cancer agents such as topotecan, cyclophosphamide, etoposide, ifosfamide

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and doxorubicin is often used for the treatment of several pediatric cancers [3–5]. VCR, an anti-mitotic agent and microtubule destabilizer extracted from the leaves of *Catharanthus roseus*, is commonly used for the treatment of multiple cancer types including ES [6]. VCR is known to induce G2-M cell cycle arrest accompanied by rise and fall of Cyclin B1 [7]. Unfortunately, VCR as well as other chemotherapeutic agents that have been used for the treatment of pediatric cancers including ES for the last five decades are associated with a multitude of adverse effects such as neuropathy and liver toxicity among others [8–12]. Despite advances in treatment modalities, the long-term side effects associated with the use of chemotherapy such as secondary malignancies, morbidity issues and relapse after treatment raise the need for innovative treatment modalities for the cure of ES [13, 14]. Thus, alternative combination treatment strategies are currently under investigation in our lab that aim to identify a safer and more effective treatment for ES.

In a recent study, we reported that ES cells expressed high levels of Specificity protein 1 (Sp1) and survivin protein levels [15]. 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. A more comprehensive analysis of Sp1 expression in cancer cell line encyclopedia (CCLE) cell lines furthers supports the overexpression of Sp1 in ES along with other pediatric as well as adult cancers (Fig. 1a). Sp1 regulates a wide array of genes, including survivin (an inhibitor of apoptosis protein) that is shown to be associated with the aggressiveness and poor prognosis of multiple cancers. Kaplan–Meier survival curves generated by R2 genomics and visualization platform and the 5 year survival was analyzed and plotted with event-free and overall survival based on survivin expression. It is evident from this analysis that high survivin expression in Ewing sarcoma correlates well with poor outcome (Fig. 1b). We and others have demonstrated that targeting Sp1 and survivin with a small molecule inhibitor, Clotam (Tolfenamic acid or TA), induces anti-proliferative activity in several adult and pediatric cancers [15–22]. TA, a non-steroidal anti-inflammatory drug (NSAID), is approved for the treatment of migraine headaches in Europe and Africa [23]. We have previously demonstrated that TA induces anti-proliferative activity of anti-neoplastic agents in adult (ovarian, colon) and pediatric cancers (neuroblastoma) [16, 17, 24].

In this investigation, we determined the efficacy of TA and VCR combination treatment against ES cells. We found that TA + VCR combination treatment caused inhibition of cell viability, induced G2/M arrest and increased apoptosis in ES cells more than either agent alone. Our results also revealed that TA alone and TA + VCR combination treatment decreased Sp1 and survivin expression, increased c-PARP levels, induced apoptosis and caused G2-M phase cell cycle arrest.

Materials and methods

Cell lines and cell culture

ES cell lines, CHLA-9 and TC-32, were obtained from the 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 4 mM L-glutamine, 1× ITS (5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL selenous acid) and fetal bovine serum. After reaching confluency, cells were passaged using puck's EDTA (140 mM NaCl, 5 mM KCl, 5.5 mM glucose, 4 mM $NaHCO_3$, 13 µM phenol red, 0.8 mM EDTA, and 9 mM HEPES, pH 7.2–7.3). All cells were cultured at 37 °C and 5% CO_2. H9C2 cells were gifted by Dr. Andras Lacko (UNTHSC Fort Worth, USA), and grown in DMEM cell culture media supplemented with 10% fetal bovine serum and maintained at 37 °C with 5% CO_2.

Chemicals and reagent

Treatment agents used in the study (TA and VCR), dimethyl sulfoxide (DMSO), and beta-actin antibody were obtained from Sigma–Aldrich (St. Louis, MO). Specificity protein 1 (Sp1) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and c-PARP antibodies were procured from Cell Signaling Technology (Danvers, MA). Survivin antibody was purchased from R&D Systems (Minneapolis, MN). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Hyclone Laboratories (Logan, Utah). ITS premix was purchased from Corning (Bedford, MA). CellTiter-Glo kit luminescent cell viability assay and caspase 3/7 assays were purchased from Promega (Madison, WI). PE-Annexin V apoptosis assay kit was obtained from BD Bioscience (San Diego, CA). Bicinchoninic acid protein assay kit and Super-Signal West Dura chemiluminescence kit used for western blot development were purchased from Pierce (Rockford, IL).

Cell viability assay

CHLA-9 and TC-32 ES cells cultured in IMDM media were treated with vehicle control (DMSO) or TA or VCR alone or combination of TA + VCR and cell viability assessment was performed using CellTiter-Glo kit (Promega, Madison, WI). Briefly, 4000 cells per well were seeded in triplicates in white walled 96-well plates (Lonza, Basel, Switzerland) and treated in with increasing concentrations of TA (10–20 µg/

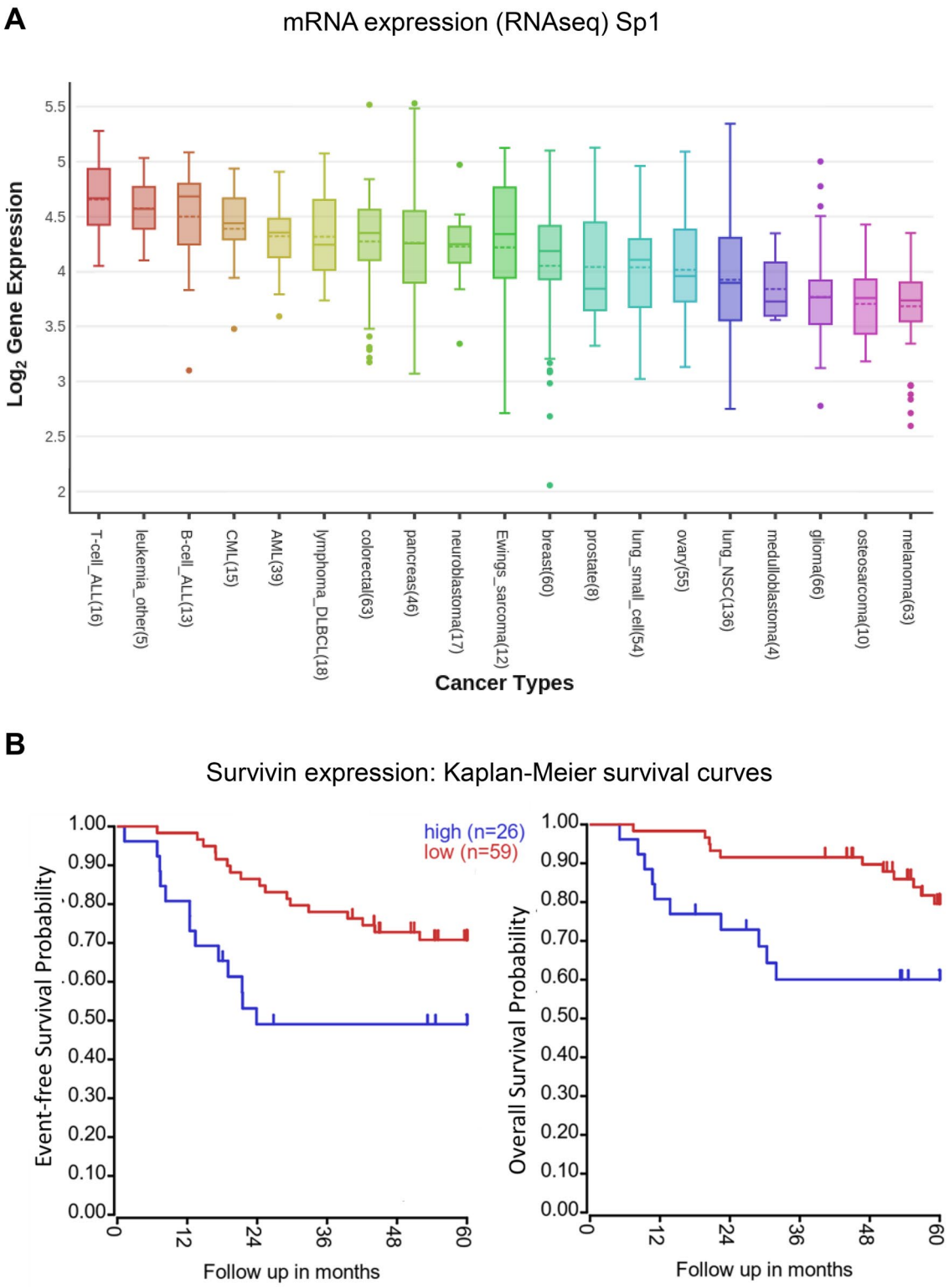


Fig. 1 Sp1 mRNA expression in human cancer cell lines and Kaplan–Meier survival curves for Survivin. **a** Sp1 expression in in various human cancer cell lines in Cancer Cell Line Encyclopedia (CCLE) dataset was obtained, analyzed and represented as log2 mRNA expression. **b** Kaplan–Meier survival curves for survivin were generated using R2 genomics and visualization platform. The 'Kaplan scan' of R2 genomics generated a Kaplan–Meier plot based on the most optimal mRNA cut-off expression levels to discriminate between a good and bad prognosis cohort. Five-year survival was analyzed and plotted with event-free and overall survival based on survivin expression. It is evident that high survivin expression in ES correlates well with worse outcome

ml) or VCR (0–2 ng/ml) for 24 h, 48 h and 72 h. Cell viability assay was carried out as per the manufacturers assay instructions. Luminescence from each well was measured on SYNERGY HT plate reader and plotted as percent cell viability versus concentration.

Caspase 3/7 assay

CHLA-9 and TC-32 cells were treated with vehicle or TA or VCR alone or TA + VCR and the caspase 3/7 activation was measured using caspase-Glo 3/7 kit (Promega, Madison, WI), according to manufacturer protocol. Briefly, 4000 cells/well were seeded in two sets (cell viability and caspases 3/7) white walled 96-well plates (Lonza, Basel, Switzerland). 24 h after plating, vehicle control (DMSO) or TA or VCR, or TA + VCR treatment was done in triplicates with optimized drug concentrations. 48 h post-treatment, the assay (CellTiter-Glo or caspase-Glo 3/7) reagent (100 µl/well) was added to measure caspase 3/7 activity luminescence was measured using SYNERGY HT plate reader. Caspase 3/7 activity was normalized to cell viability and the data were presented as mean ± SD.

Annexin V apoptosis assay

ES cells were harvested after treatment with vehicle (DMSO) or drug alone (TA or VCR), or combination (TA + VCR) for 48 h. Cells were washed with 1× PBS and stained with PE-Annexin V/7-AAD apoptosis kit (BD Biosciences) to measure apoptotic cells according to manufactures protocol. Data was acquired using FC500 flow cytometer and analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR).

Propidium iodide (PI) cell cycle analysis

CHLA-9 and TC-32 cells wereseeded in 6 wells plates treated with vehicle or TA (15 µg/ml) or VCR (0.5 ng/ml) or TA (15 µg/ml) + VCR (0.5 ng/ml) and, processed for cell cycle phase distribution analysis. Cells were harvested at 12 h and 24 h post-treatment, washed with PBS and fixed in 1 ml cold 70% ethanol in water for overnight at 4 °C. Upon fixation cells were stained with (0.20 µg/ml PI and 20 µg/ml RNAse A in 1× PBS), and incubated at room temperature for 15 min. Data was acquired on FC500 flow cytometer and analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR). Results were represented as cell count versus PI intensity (DNA content).

Western immunoblotting

CHLA-9 and TC-32 cells were cultured in 100 mm cell culture dishes and treated with vehicle control (DMSO) or TA or VCR or combination of TA + VCR. 48 h post-treatment cells were harvested, expression of proteins of interest in whole cell lysates were evaluated using western blot analysis as previously described [18, 24].

Kaplan–Meier patient survival curves

Kaplan–Meier patient survival curves for Survivin (BIRC5) mRNA expression were generated by using R2 genomics and visualization platform. Ewing sarcoma database (tumor Ewing sarcoma [Core Exon]-Dirksen-85) with survival information was chosen for analysis. The 'Kaplan scan' of R2 genomics generates a Kaplan–Meier Plot based on the most optimal mRNA cut-off expression levels to discriminate between a good and bad prognosis cohort ($p < 0.05$). Five-year survival was analyzed and plotted with event-free and overall survival based on survivin mRNA expression.

Data analysis

All experiments were performed in triplicates. Statistical data analysis was performed by one-way ANOVA at a 95% confidence interval using GraphPad Prism 6.0. Comparison were made between untreated (vehicle) and treatment groups. For signfucance, p-value < 0.05 was considered.

Results

Effect of TA and VCR on ES cell viability

The anti-proliferative activity of TA and VCR was investigated using CHLA-9 and TC-32 cell lines. To obtain the working concentrations for TA and VCR, ES cell were treated with increasing concentrations of TA (0–20 µg/ml) or VCR (0–2 ng/ml), and cell viabilities were evaluated at 24, 48 and 72 h post-treatment. Results showed that both TA and VCR treatment resulted in a time- and dose-dependent cell viability inhibition in ES cell lines (Fig. 2). The derived IC_{50} values at 48 h for these cell lines were ranged between 12 and 15 µg/ml for TA, and that of VCR ranged between 0.35 and 0.63 ng/ml. These results suggest that sensitivity of ES cells to clinical/therapeutic doses of TA and VCR.

To determine if combining these drugs enhances anti-proliferative activity, CHLA-9 and TC-32 cells were treated with 10 or 15 µg/ml TA or 0.5 ng/ml VCR or TA 10 µg/ml + VCR 0.5 n/ml or TA 15 µg/ml + VCR 0.5 ng/ml, and cell viability was measured after 48 h. When compared to agent alone, the combination of the agents under investigation showed significantly higher (growth-inhibitory) response in both cell lines (Fig. 3a, b). After analyzing the results, 15 µg/ml of TA and 0.5 ng/ml of VCR were chosen for further studies unless otherwise noted.

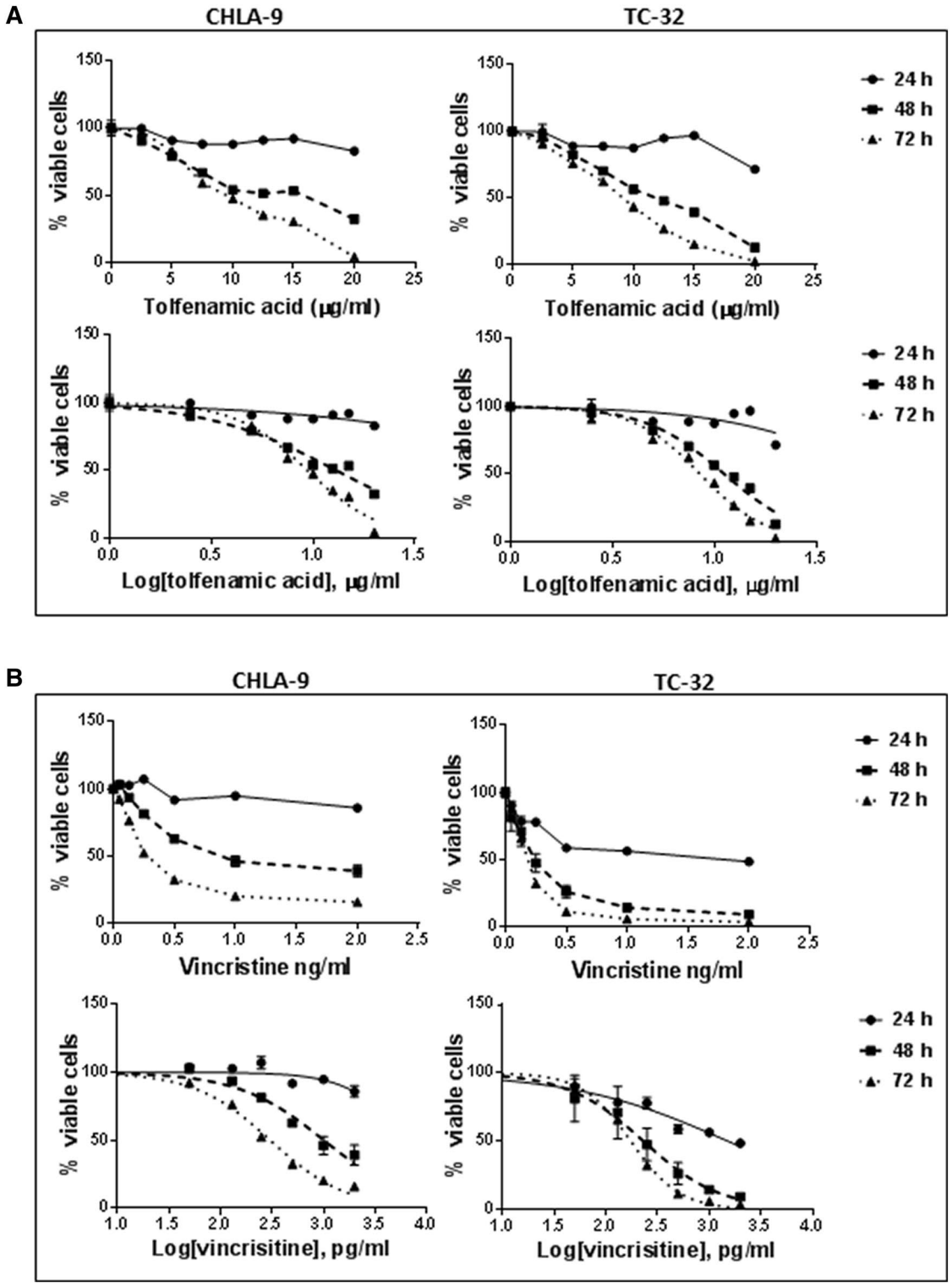


Fig. 2 TA and VCR decreased cell viability of ES cells in a dose- and time-dependent manner. Human ES cells, CHLA-9 and TC-32 were treated with DMSO (control) or increasing concentrations (0–20 µg/ml) of TA and (0–5 ng/ml) of VCR, and the cell viability assay was performed using CellTiter-Glo kit (Promega) at 24 h, 48 h and 72 h post-treatment. Data shown for **a** TA and **b** VCR indicate the percent cell viability normalized to control (DMSO). Each data point represents the mean ± SD of triplicate determinations

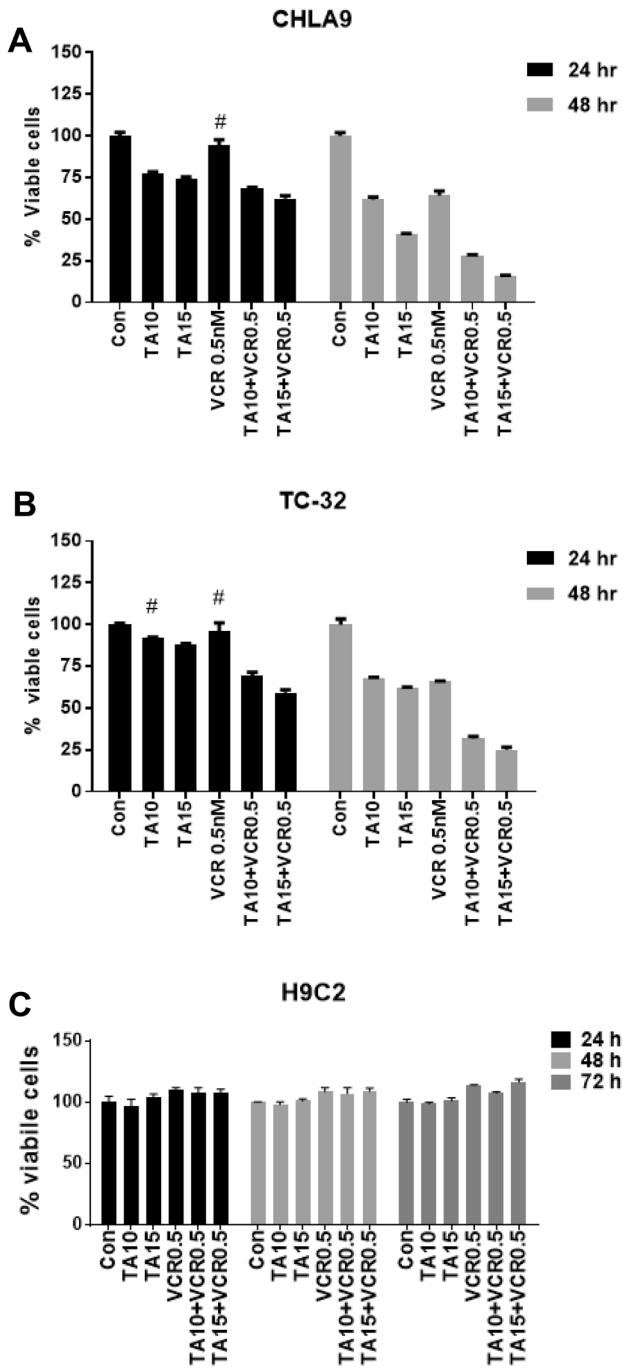


Fig. 3 Combination treatment of TA and VCR inhibited ES cell viability but not cardiomyocytes. ES cell lines, **a** CHLA-9 and **b** TC-32 and **c** cardiomyocytes H9C2 were treated with DMSO (control) or TA (10 or 15 µg/ml) or VCR (0.5 ng/ml) or combination of TA + VCR. 48 h post-treatment cell viability 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. Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0. **a, b** All values were significantly different (p value < 0.05) excluding the bars marked with #. **c** There was no difference between control and treated samples in H9C2 cells. *CON* control (DMSO), *TA 10* tolfenamic acid 10 µg/ml, *TA 15* tolfenamic acid 15 µg/ml, *VCR0.5* vincristine 0.5 ng/ml, *TA10 + VCR 0.5* tolfenamic acid 15 µg/ml + vincristine 0.5 ng/ml, *TA15 + VCR 0.5* tolfenamic acid 15 µg/ml + vincristine 0.5 ng/ml

Effect of TA and VCR on 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 studied the effect of TA and VCR as single agents or as a combination treatment of TA + VCR on H9C2 cardiomyocyte cells. H9C2 cells were treated with DMSO (vehicle), TA (10 µg/ml or 15 µg/ml), VCR (0.5 ng/ml) alone or combination of TA + VCR, and cell viabilities were measured at 24 h, 48 h and 72 h post-treatments. Results showed that TA alone, VCR alone or TA + VCR combination treatment do not induce toxicity in H9C2 cells (Fig. 3c).

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

CHAL-9 and TC-32 ES cells were treated with optimized doses of TA (10 and 15 µg/ml) and VCR (0.5 ng/ml) or TA + VCR combination. Sp1 and survivin protein expression levels were assayed using western immunoblot analysis. The results showed that TA + VCR combination treatment significantly inhibited both Sp1 and survivin as compared to the TA or VCR alone. Interestingly, the inhibition of cell viability was accompanied by decreased Sp1 and survivin protein expression at 48 h post-treatment (Fig. 4a).

Effect of TA and VCR on apoptosis in ES cells

ES cells, CHLA-9 and TC-32, were treated with TA and VCR alone or in combination. The effect of TA and VCR on apoptosis was studied by performing Annexin V flow cytometry and measuring the apoptosis markers, caspase 3/7 activation and c-PARP protein level. The caspase 3/7 activity levels were significantly increased upon TA + VCR combination treatment when compared to TA or VCR alone (Fig. 4b). A similar trend was observed in c-PARP protein expression (Fig. 4c). Flow cytometry results using Annexin V staining also showed higher percentage of apoptotic or dead cells and low percentage of live cells in TA + VCR treated group compared to untreated (Fig. 5). These results indicate that VCR-induced anti-proliferative effect in the presence of TA is accompanied by an increase in apoptosis.

Effect of TA and VCR on cell cycle phase distribution

The combination treatment of TA + VCR dramatically decreased cell proliferation in ES cells. 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 assess the TA and VCR alone or TA + VCR

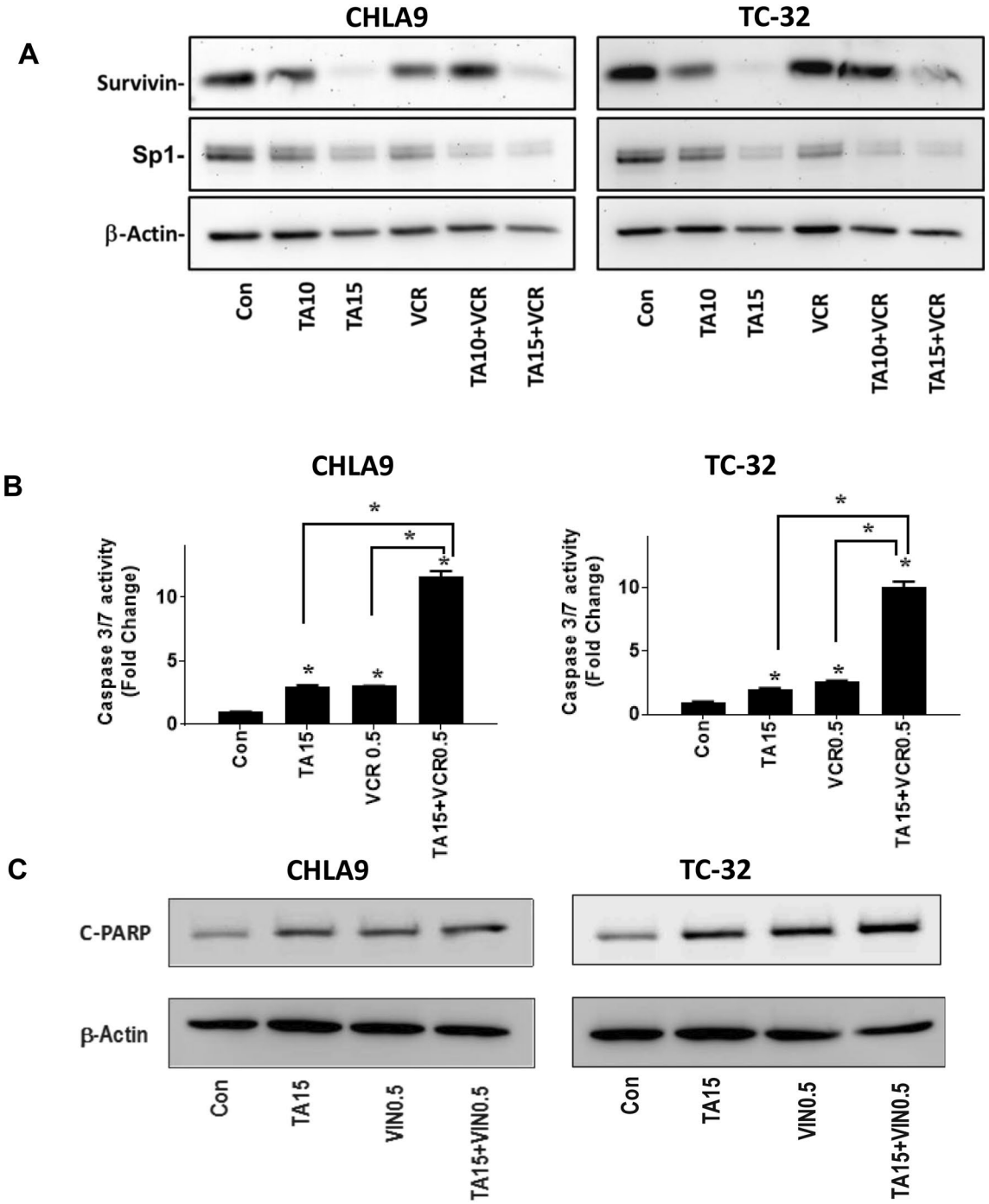


Fig. 4 Combination treatment of TA and VCR inhibited Sp1 and survivin protein expression and up-regulated apoptotic markers, c-PARP expression and caspase 3/7 activity. CHLA-9 and TC-32 cells were treated with treated with DMSO (control) or TA (10 or 15 μg/ml) or VCR (0.5 ng/ml) or combination of TA + VCR. 48 h post-treatment, **a** whole cell lysates were prepared 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. **b** Caspase 3/7 activity was measured using caspase 3/7 Glo kit. **c** Whole cell lysates were used to determine the expression of c-PARP by Western blot analysis. The data were obtained from three independent experiments. Representative Western blot images are shown in the figure. Statistical analysis was performed by one-way ANOVA using GraphPad Prism V6.0. Bars marked with * are significantly (p value < 0.05) different from Control or the indicated bars. *CON* control (DMSO), *TA 10* tolfenamic acid 10 μg/ml, *TA 15* tolfenamic acid 15 μg/ml, *VCR0.5* vincristine 0.5 ng/ml, *TA10 + VCR 0.5* tolfenamic acid 15 μg/ml + vincristine 0.5 ng/ml, *TA15 + VCR 0.5* tolfenamic acid 15 μg/ml + vincristine 0.5 ng/ml

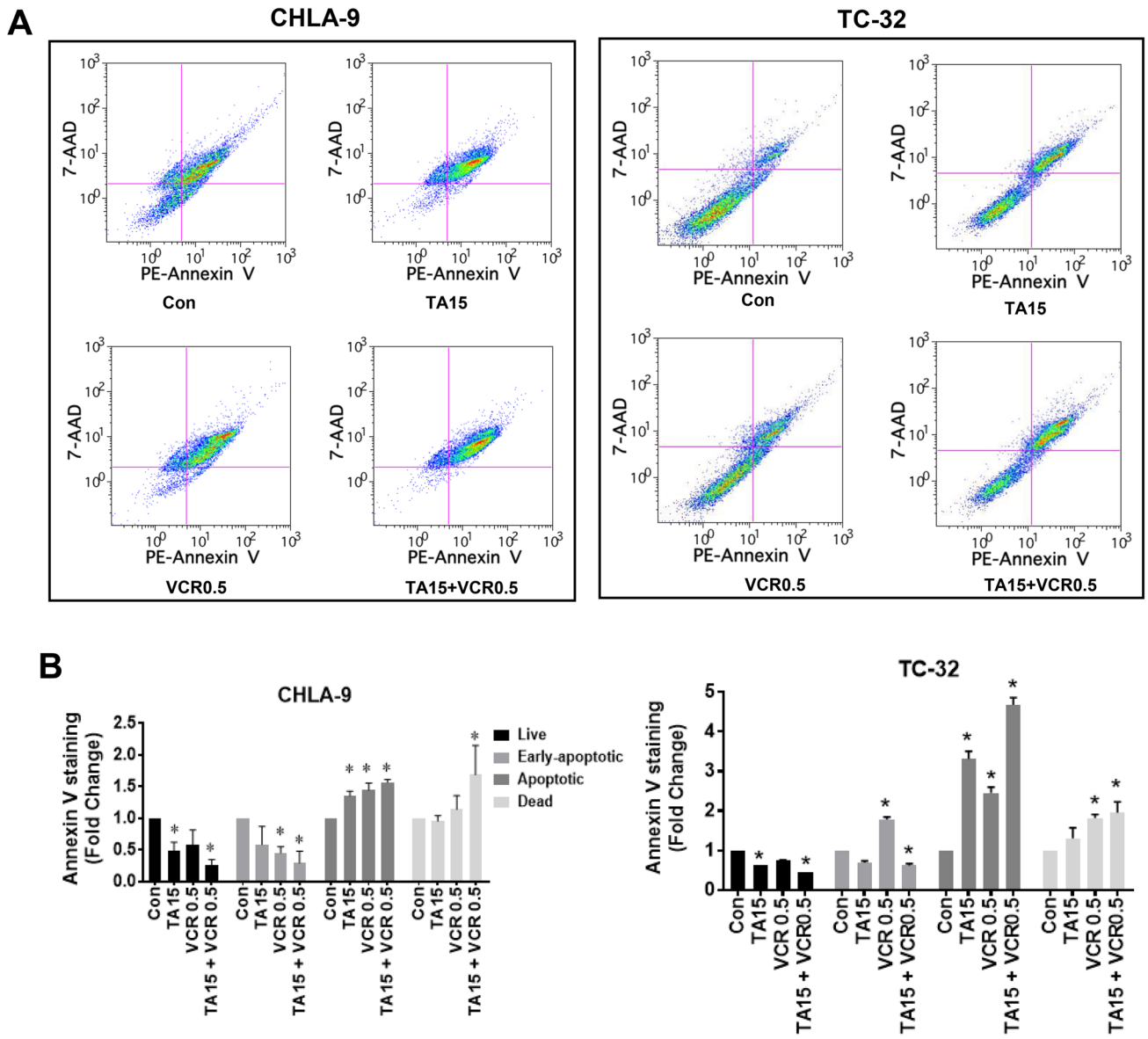
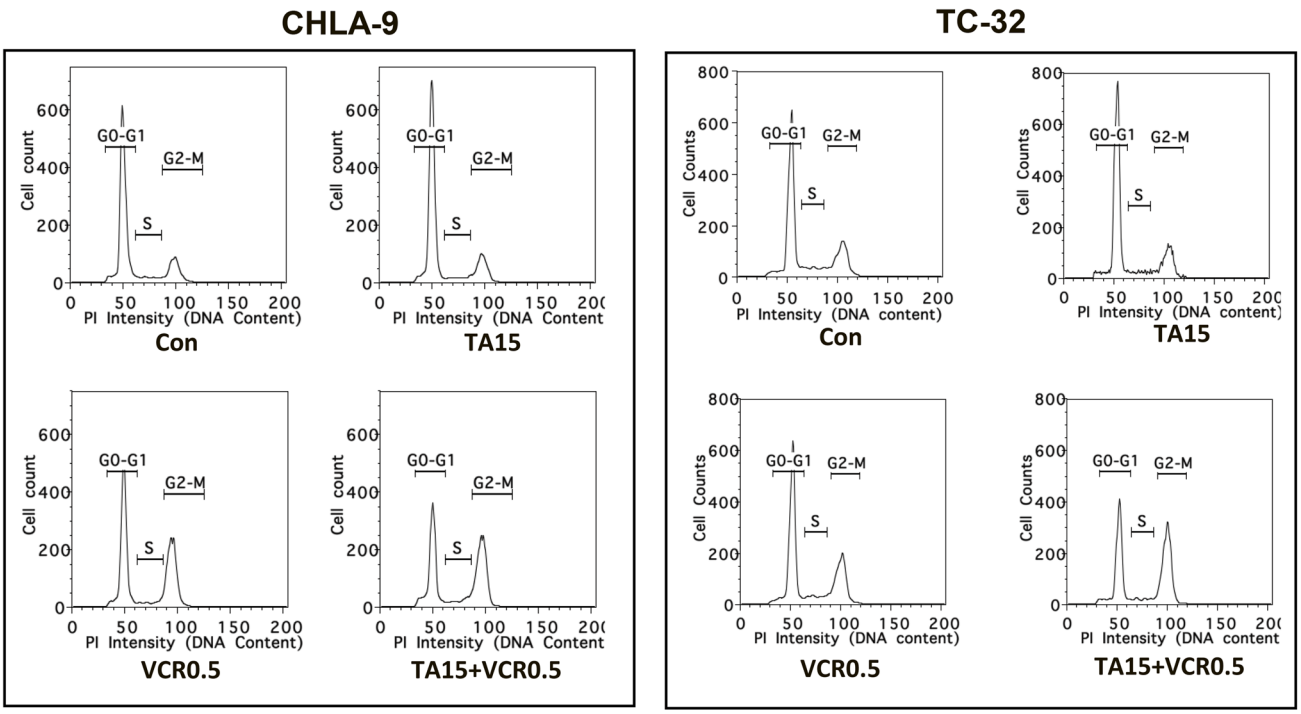


Fig. 5 Combination treatment with TA and VCR induced apoptotic cell population. CHLA-9 and TC-32 cells were treated with DMSO or TA (15 µg/ml) or VCR (0.5 ng/ml) or TA + VCR combination. 48 h post-treatment, apoptotic cell population was analyzed using Annexin V-PE/7-AAD kit on BD LSR II (BD Bioscience). **a** Representative Annexin V/7-AAD plots for various treatments are shown. **b** The fold change over control in each quarter was from three independent experiments were used to compare the results among single or combination treatment. The graphs represent mean ± SD and the Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0 (* represents statistical significance compared to control; p value < 0.05). *CON* control (DMSO), *TA 15* tolfenamic acid 15 µg/ml, *VCR0.5* vincristine 0.5 ng/ml, *TA15 + VCR 0.5* tolfenamic acid 15 µg/ml + vincristine 0.5 ng/ml

treatment induced cell cycle changes. TA has been shown to induce G0/G1 cell cycle arrest in neuroblastoma cells [25]. Consistent with previous results, TA induced G0/G1 cell cycle arrest in ES cells. The mechanism of action of VCR includes destabilization of microtubule formation followed by G2/M arrest [6]. VCR treatment significantly increased G2/M arrest in CHLA-9 cells (VCR 45.5% ± 3.8; TA + VCR: 48.7 ± 2.7) and TC-32 cells (VCR: 26.8% ± 0.7; TA + VCR: 49.3 ± 2.7) as early as early as 12 h after treatment (Supplementary data Figs. S1&2) and sustained through 24 h (Fig. 6).

Discussion

ES is the second most common tumor of bone and soft tissue that affects adolescents and young adults. VCR, a Vinca alkaloid, is an anti-neoplastic agent that is currently used



CHLA-9

	Con	TA15	VCR0.5	TA15+VCR0.5
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*

TC-32

	Con	TA15	VCR0.5	TA15+VCR0.5
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*

Fig. 6 Combination treatment of TA and VCR caused G2/M cell cycle arrest. CHLA-9 and TC-32 cells were treated with DMSO or TA (15 µg/ml) or VCR (0.5 ng/ml) or combinations of TA + VCR. Cell cycle phase distribution was assayed at 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 below. 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 statistical significance compared to control with p value < 0.05). *CON* control (DMSO), *TA 15* tolfenamic acid 15 µg/ml, *VCR0.5* vincristine 0.5 ng/ml, *TA15 + VCR 0.5* tolfenamic acid 15 µg/ml + vincristine 0.5 ng/ml

in FDA approved combination treatment regimens to treat several adult and pediatric malignancies including ES [3, 26–29]. However, its applications are limited due to peripheral neuropathy associated with its use. This VCR-induced peripheral neuropathy (VIPN), although undetectable in early stages, severely affects the quality of long-term survivors [30–32]. 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, TA, to induce anti-proliferative activity of VCR in ES cells. Our lab and others have previously showed that TA induces anti-proliferative activity in several types of cancer

cells by inhibiting Sp1 and survivin protein expression including in ES cells [15, 18, 25, 33, 34]. Our results showed TA and VCR 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 (Figs. 2, 3). Interestingly, TA or VCR or combination have no significant effect on cytotoxicity of H9C2 cells. Cancer cells overexpress Sp1 and survivin and both of these markers enhance cell survival and reduce apoptosis. TA inhibits both Sp1 and survivin thereby causing anti-proliferative effect in cancer cells. Cardiomyocytes express low levels of Sp1 and survivin. The doses of TA tested in this study may not inhibit Sp1 and survivin in H9C2 cells and therefore TA or the combination treatment is not causing cytotoxicity in these cells. Similarly, we demonstrated before that TA (up to 50 μM) has no cytotoxic effect on non-malignant cells [33]. It is in fact very encouraging to further consider this combination for clinical application.

Survivin, an inhibitor of apoptosis protein, is upregulated in several cancers including sarcomas [35]. Kaplan–Meier survival curves were generated by using R2 genomics and visualization platform further confirms the association of higher expression of survivin with low survival rates when compared to low expression of survivin in ES. Previous reports suggest that Sp1 transcription factor regulates survivin gene expression by modulating its promoter activity [36]. Our laboratory and others demonstrated that TA induces proteasome mediated degradation and alters the DNA binding activity of Sp1 transcription factor in cancer cells [15, 37]. Most importantly, we showed that anti-proliferative activity of TA may be linked to its ability to inhibit survivin protein expression as found in several cancer types [15, 18, 19, 25, 38]. Results of our investigation revealed that TA + VCR combination treatment inhibited both Sp1 and survivin protein expression (Fig. 4a). Although there are no previous reports of the modulation of Sp1 in response to VCR treatment, our results revealed that VCR treatment caused a moderate downregulation of Sp1 in CHLA-9 cells but not in TC-32 cells (Fig. 3). Previously, VCR treatment was shown to upregulate survivin protein expression in chromic myeloid leukemia cells [39]. However, recent report suggests that VCR enhanced the anti-cancer activity of cotylenin A partially by inhibiting expression of anti-apoptotic proteins, cellular inhibitor of apoptosis protein (cIAP), and survivin [40]. Survivin regulates G2/M cell cycle phase transition [41]. Therefore, the effect of VCR, which causes G2/M cell cycle arrest, on survivin expression could depend upon the underlying cellular and genetic context and needs to be further studied in details.

The anti-proliferative activity of TA and VCR, alone or in combination, could be linked to activation of apoptosis pathways. Caspases, proteins that cleave 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 (dependent upon mitochondrial cytochrome *C* mediated activation) apoptosis pathways [42–44]. VCR and cisplatin, a microtubule disrupting drugs, is shown to 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 [45]. We previously showed that TA induces apoptosis in human neuroblastoma cells via activation of both caspase 3/7 and caspase 8/9 [24]. Several anti-cancer agents can induce apoptosis in cancer cells by increasing caspase 3/7 activity [46]. Annexin V flow cytometry results showed that TA + VCR combination treatment significantly increased apoptotic cells compared to control and TA or VCR single agent. In this study, our results revealed that combination treatment with TA + VCR treatment significantly increased caspase 3/7 activity which was accompanied by elevated c-PARP protein levels in whole cell lysate (Fig. 4). Furthermore, these results strongly correlated with Annexin V flow cytometry results (Fig. 5). This supports our hypothesis that TA and VCR combination treatment could synergistically induce activating caspases (caspase 8 and caspase 9) and thereby resulting in dramatic increase in effector caspase, caspase 3/7, activation in ES cells.

As discussed earlier, anti-proliferative activity of TA + VCR combination treatment could involve activation of both apoptosis as well as cell cycle arrest. TA induces G0/G1 cell cycle arrest in neuroblastoma cells [25], whereas, VCR can induce G2/M arrest in several cancer types [47]. In this investigation, we assessed the TA and VCR single agent or TA + VCR combination treatment induced alterations in cell cycle phase distribution in ES cells at 12 h (Supplementary data Figs. S1&2) and 24 h (Fig. 6). Our results revealed that TA + VCR combination treatment caused G2/M arrest in ES cells (Fig. 6). Given the differences in the mechanism of action, it is possible that VCR treatment induced cell cycle arrest could precede that of TA, and could predominate in TA + VCR combination treatment. These observations were consistent with previous reports and cell cycle phase distribution observations [48, 49].

Using pre-clinical mouse model, we and others shown that TA is safe to use and did not cause any apparent toxicity to vital organs [33, 50]. NSAIDs are reported to cause cardiotoxicity. In this study, we assessed the effect of single agent or TA + VCR combination treatment on cell viability of H9C2 cells. Our results indicate that TA alone and TA + VCR do not cause any toxicity in cardiomyocyte (Fig. 3). TA is approved by the 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

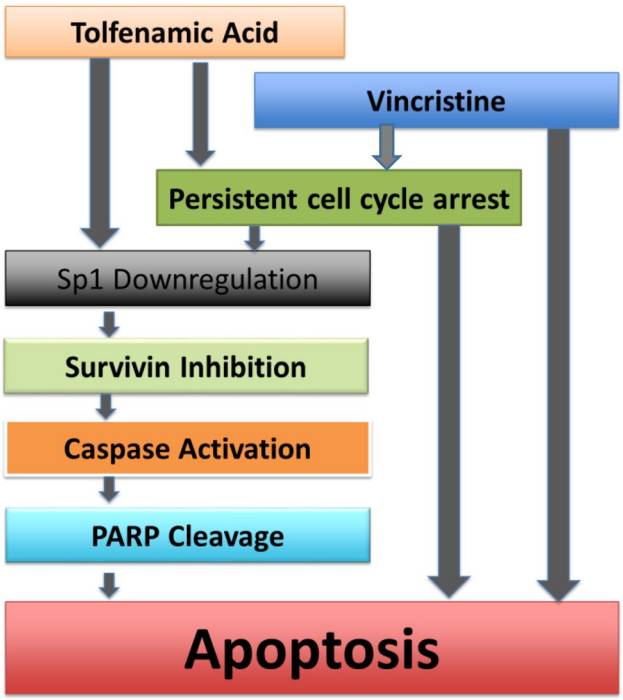


Fig. 7 The proposed activity of combination treatment: TA, enhances the growth inhibition of TA and VCR in ES cells by inhibiting Sp1 and survivin expression. TA + VCR combination treatment caused significant activation of caspase 3/7, increased c-PARP expression and induced apoptosis potentially activation apoptotic mechanisms

in colon cancer cells [16, 24]. 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 summary, this study shows that clotam is potentially acting as a sensitizing agent to enhances the anti-proliferative response of VCR in ES cells by inhibiting Sp1 and survivin expression. TA + VCR combination treatment caused significant activation of caspase 3/7, increased c-PARP expression and induced apoptosis (Fig. 7). Among the two ES cell lines, TC-32 cells had higher sensitivity for TA + VCR combination treatment. TA + VCR caused higher anti-proliferative response in TC-32 cells as seen in Annexin V apoptosis and cell cycle analysis than in CHLA-9 cells. VCR alone or in the presence of TA caused G2/M arrest in both ES cells (Fig. 7). Importantly, TA or VCR or TA + VCR did not cause any cytotoxicity in H9C2 cardiomyocytes supporting the safety against non-malignant cells. Taken together, these results from our investigation provides the rational for development of novel combination treatments to enhance efficacy of VCR in combination with TA for the treatment of ES.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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