Published in final edited form as:

Apoptosis. 2019 February ; 24(1-2): 21–32. doi:10.1007/s10495-018-1508-1.

Clotam Enhances Anti-Proliferative Effect of Vincristine in Ewing Sarcoma Cells

Sagar Shelake^{1,#}, Umesh T. Sankpal¹, Don Eslin², W. Paul Bowman^{1,3}, Jerry W. Simecka⁴, Sangram Raut⁵, Anish Ray³, and Riyaz Basha^{1,2,3,*}

¹Department of Pediatrics, Texas College of Osteopathic Medicine, University of North Texas Health Science Center, Fort Worth, Texas, 76107

²Arnold Palmer Hospital for Children, Orlando, FL 32806

³Hematology and Oncology, Cook Children's Medical Center, Fort Worth, TX 76104

⁴Pre-clinical Services, UNT Systems College of Pharmacy, University of North Texas Health Science Center, Fort Worth, Texas, 76107

⁵Department of Physiology and Anatomy, University of North Texas Health Science Center, Fort Worth, Texas, 76107

Abstract

Objective: 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).

Methods: 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 hours. Effect of TA 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.

Results: 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.

*Correspondence: Riyaz Basha, Ph.D., University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX, 76107, Tel: +1-817-735-0302; Fax: +1-817-735-2118, riyaz.basha@unthsc.edu.

#Current address: Johns Hopkins University School of Medicine, Baltimore, MD 21287.

CONFLICT OF INTEREST:

None. Authors SS, UTS, DE, WPB, JWS, AR and RB have no conflict of interest.

Conclusion: 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 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 further supports the overexpression of Sp1 in ES along with other pediatric as well as adult cancers (Figure 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 five 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 in poor outcome (Figure 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.

MATERIAL 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 4mM L-Glutamine, 1X 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 pucks EDTA (140 mM NaCl, 5 mM 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). All cells were cultured at 37°C and 5% CO₂. 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% CO₂.

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/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, 4,000 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 μ l/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 $\pm$ 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 1X 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 μ g/ml) or VCR (0.5 ng/ml) or TA (15 μ g/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 4⁰ C. Upon fixation cells were stained with (0.20 μ g/ml PI and 20 μ g/ml RNase A in 1X 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 vs 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 significance, 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 $\mu\text{g/ml}$) 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 (Figure 2). The derived IC_{50} values at 48 h for these cell lines were ranged between 12–15 $\mu\text{g/ml}$ for TA, and that of VCR ranged between 0.35–0.63 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 $\mu\text{g/ml}$ TA or 0.5 ng/ml VCR or TA 10 $\mu\text{g/ml}$ + VCR 0.5 ng/ml or TA 15 $\mu\text{g/ml}$ + 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 (Figure 3A&B). After analyzing the results, 15 $\mu\text{g/ml}$ of TA and 0.5 ng/ml of VCR were chosen for further studies unless otherwise noted.

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 $\mu\text{g/ml}$ or 15 $\mu\text{g/ml}$), 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 (Figure 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 $\mu\text{g/ml}$) 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 (Figure 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 (Figure 4B). A similar trend was observed in c-PARP protein expression (Figure 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 (Figure 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 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 $45.5\% \pm 3.8$; TA+VCR: 48.7 ± 2.7) and TC-32 cells (VCR: $26.8\% \pm 0.7$; TA+VCR: 49.3 ± 2.7) as early as early as 12 h after treatment (Supplementary data Figures S1&2) and sustained through 24 h (Figure 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 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 antineoplastic 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 (Fig. 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 μ M) 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 (Figure 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 (Figure 3). Previously, VCR treatment was shown to upregulate survivin protein expression in chronic 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 (Figure 4). Furthermore, these results strongly correlated with Annexin V flow cytometry results (Figure 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 Figures S1&2) and 24 h (Figure 6). Our results revealed that TA+VCR combination treatment caused G2/M arrest in ES cells (Figure 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 (Figure 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 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 (Figure 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 (Figure 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS:

This work is partially supported by HyundaiHopeOnWheels Young Investigator Grant awarded to AR. RB is supported by National Institute for Minority Health and Health Disparities (grant#: 2U54 MD006882-06) Authors

thank Childhood Cancer Repository at the Texas Tech University Health Sciences Center in Lubbock, TX for providing Ewing Sarcoma cell lines.

REFERENCE:

1. Jo VY, Fletcher CD. (2014) WHO classification of soft tissue tumours: an update based on the 2013 (4th) edition. *Pathology* 46:95–104. [PubMed: 24378391]
2. Jemal A, Siegel R, Xu J, Ward E. (2010) Cancer statistics, 2010. *CA Cancer J Clin* 60:277–300.
3. Mascarenhas L, Felgenhauer JL, Bond MC, et al. (2016) Pilot Study of Adding Vincristine, Topotecan, and Cyclophosphamide to Interval-Compressed Chemotherapy in Newly Diagnosed Patients With Localized Ewing Sarcoma: A Report From the Children's Oncology Group. *Pediatr Blood Cancer* 63:493–498. [PubMed: 26579879]
4. Kebudi R, Cakir FB, Gorgun O, Agaoglu FY, Darendeliler E. (2013) A modified protocol with vincristine, topotecan, and cyclophosphamide for recurrent/progressive ewing sarcoma family tumors. *Pediatr Hematol Oncol* 30:170–177. [PubMed: 23484903]
5. Ferrari S, Palmerini E, Alberghini M, et al. (2010) Vincristine, doxorubicin, cyclophosphamide, actinomycin D, ifosfamide, and etoposide in adult and pediatric patients with nonmetastatic Ewing sarcoma. Final results of a monoinstitutional study. *Tumori* 96:213–218. [PubMed: 20572576]
6. Gaspar N, Hawkins DS, Dirksen U, et al. (2015) Ewing Sarcoma: Current Management and Future Approaches Through Collaboration. *Journal of Clinical Oncology* 33:3036–U3140. [PubMed: 26304893]
7. Nigg EA. (2001) Mitotic kinases as regulators of cell division and its checkpoints. *Nature reviews Molecular cell biology* 2:21–32. [PubMed: 11413462]
8. Yap BS, Baker LH, Sinkovics JG, et al. (1980) Cyclophosphamide, vincristine, adriamycin, and DTIC (CYVADIC) combination chemotherapy for the treatment of advanced sarcomas. *Cancer Treat Rep* 64:93–98. [PubMed: 7379060]
9. Breitfeld PP, Lyden E, Raney RB, et al. (2001) Ifosfamide and etoposide are superior to vincristine and melphalan for pediatric metastatic rhabdomyosarcoma when administered with irradiation and combination chemotherapy: a report from the Intergroup Rhabdomyosarcoma Study Group. *J Pediatr Hematol Oncol* 23:225–233. [PubMed: 11846301]
10. Thompson J, George EO, Poquette CA, et al. (1999) Synergy of topotecan in combination with vincristine for treatment of pediatric solid tumor xenografts. *Clin Cancer Res* 5:3617–3631. [PubMed: 10589779]
11. Simsek T, Uner M, Trak B, Erman O, Zorlu GC. (1998) Toxicity of chemotherapeutical protocols in the treatment of uterine sarcomas (Vincristine, actinomycin D, Cyclophosphamide VAC versus ifosfamide). *Eur J Gynaecol Oncol* 19:405–407. [PubMed: 9744738]
12. Paulussen M, Ahrens S, Craft AW, et al. (1998) Ewing's tumors with primary lung metastases: survival analysis of 114 (European Intergroup) Cooperative Ewing's Sarcoma Studies patients. *J Clin Oncol* 16:3044–3052. [PubMed: 9738574]
13. Bacci G, Picci P, Ferrari S, et al. (1998) Neoadjuvant chemotherapy for Ewing's sarcoma of bone: no benefit observed after adding ifosfamide and etoposide to vincristine, actinomycin, cyclophosphamide, and doxorubicin in the maintenance phase--results of two sequential studies. *Cancer* 82:1174–1183. [PubMed: 9506366]
14. Craft A, Cotterill S, Malcolm A, et al. (1998) Ifosfamide-containing chemotherapy in Ewing's sarcoma: The Second United Kingdom Children's Cancer Study Group and the Medical Research Council Ewing's Tumor Study. *J Clin Oncol* 16:3628–3633. [PubMed: 9817284]
15. Shelake S, Sankpal UT, Paul Bowman W, Wise M, Ray A, Basha R. (2016) Targeting specificity protein 1 transcription factor and survivin using tolfenamic acid for inhibiting Ewing sarcoma cell growth. *Invest New Drugs*.
16. Sankpal UT, Nagaraju GP, Gottipolu SR, et al. (2016) Combination of tolfenamic acid and curcumin induces colon cancer cell growth inhibition through modulating specific transcription factors and reactive oxygen species. *Oncotarget* 7:3186–3200. [PubMed: 26672603]

17. Sankpal UT, Ingersoll SB, Ahmad S, et al. (2016) Association of Sp1 and survivin in epithelial ovarian cancer: Sp1 inhibitor and cisplatin, a novel combination for inhibiting epithelial ovarian cancer cell proliferation. *Tumour Biol* 37:14259–14269. [PubMed: 27581819]
18. Basha R, Connelly SF, Sankpal UT, et al. (2016) Small molecule tolfenamic acid and dietary spice curcumin treatment enhances antiproliferative effect in pancreatic cancer cells via suppressing Sp1, disrupting NF-kB translocation to nucleus and cell cycle phase distribution. *J Nutr Biochem* 31:77–87. [PubMed: 27133426]
19. Sutphin RM, Connelly SF, Lee CM, et al. (2014) Anti-leukemic response of a NSAID, tolfenamic acid. *Target Oncol* 9:135–144. [PubMed: 23609055]
20. Pathi S, Li X, Safe S. (2014) Tolfenamic acid inhibits colon cancer cell and tumor growth and induces degradation of specificity protein (Sp) transcription factors. *Mol Carcinog* 53 Suppl 1:E53–61. [PubMed: 23670891]
21. Chang JW, Kang SU, Choi JW, et al. (2014) Tolfenamic acid induces apoptosis and growth inhibition in anaplastic thyroid cancer: Involvement of nonsteroidal anti-inflammatory drug-activated gene-1 expression and intracellular reactive oxygen species generation. *Free Radic Biol Med* 67:115–130. [PubMed: 24216474]
22. Kim HJ, Cho SD, Kim J, et al. (2013) Apoptotic effect of tolfenamic acid on MDA-MB-231 breast cancer cells and xenograft tumors. *J Clin Biochem Nutr* 53:21–26. [PubMed: 23874066]
23. Vaitkus A, Pauza V. (2002) [Effectiveness of tolfenamic acid in the prevention of migraine]. *Medicina (Kaunas)* 38:296–303. [PubMed: 12474702]
24. Shelake S, Eslin D, Sutphin RM, et al. (2015) Combination of 13 cis-retinoic acid and tolfenamic acid induces apoptosis and effectively inhibits high-risk neuroblastoma cell proliferation. *Int J Dev Neurosci* 46:92–99. [PubMed: 26287661]
25. Eslin D, Sankpal UT, Lee C, et al. (2013) Tolfenamic acid inhibits neuroblastoma cell proliferation and induces apoptosis: a novel therapeutic agent for neuroblastoma. *Mol Carcinog* 52:377–386. [PubMed: 22213339]
26. Dittus C, Grover N, Ellsworth S, Tan X, Park SI. (2018) Bortezomib in combination with dose-adjusted EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) induces long-term survival in patients with plasmablastic lymphoma: a retrospective analysis. *Leuk Lymphoma*:1–7.
27. Qaddoumi I, Billups CA, Tagen M, et al. (2012) Topotecan and vincristine combination is effective against advanced bilateral intraocular retinoblastoma and has manageable toxicity. *Cancer* 118:5663–5670. [PubMed: 22516936]
28. Isono M, Sato A, Asano T. (2016) A Case of Long-term Survival of Advanced Paratesticular Rhabdomyosarcoma Treated With a Multimodal Therapy Including a Combination of Cyclophosphamide, Vincristine, Doxorubicin and Dacarbazine. *Urol Case Rep* 7:3–6. [PubMed: 27335778]
29. Wagner MJ, Gopalakrishnan V, Ravi V, et al. (2017) Vincristine, Ifosfamide, and Doxorubicin for Initial Treatment of Ewing Sarcoma in Adults. *Oncologist* 22:1271–1277. [PubMed: 28710342]
30. Lavoie Smith EM, Li L, Chiang C, et al. (2015) Patterns and severity of vincristine-induced peripheral neuropathy in children with acute lymphoblastic leukemia. *J Peripher Nerv Syst* 20:37–46. [PubMed: 25977177]
31. Mora E, Smith EM, Donohoe C, Hertz DL. (2016) Vincristine-induced peripheral neuropathy in pediatric cancer patients. *Am J Cancer Res* 6:2416–2430. [PubMed: 27904761]
32. Grisold W, Cavaletti G, Windebank AJ. (2012) Peripheral neuropathies from chemotherapeutics and targeted agents: diagnosis, treatment, and prevention. *Neuro Oncol* 14 Suppl 4:iv45–54. [PubMed: 23095830]
33. Sankpal UT, Lee CM, Connelly SF, et al. (2013) Cellular and organismal toxicity of the anti-cancer small molecule, tolfenamic acid: a pre-clinical evaluation. *Cell Physiol Biochem* 32:675–686. [PubMed: 24030139]
34. Eslin D, Lee C, Sankpal UT, et al. (2013) Anticancer activity of tolfenamic acid in medulloblastoma: a preclinical study. *Tumour Biol* 34:2781–2789. [PubMed: 23686785]

35. Kappler M, Kotzsch M, Bartel F, et al. (2003) Elevated expression level of survivin protein in soft-tissue sarcomas is a strong independent predictor of survival. *Clin Cancer Res* 9:1098–1104. [PubMed: 12631613]
36. Xu R, Zhang P, Huang J, Ge S, Lu J, Qian G. (2007) Sp1 and Sp3 regulate basal transcription of the survivin gene. *Biochem Biophys Res Commun* 356:286–292. [PubMed: 17350596]
37. Abdelrahim M, Baker CH, Abbruzzese JL, Safe S. (2006) Tolfenamic acid and pancreatic cancer growth, angiogenesis, and Sp protein degradation. *J Natl Cancer Inst* 98:855–868. [PubMed: 16788159]
38. Basha R, Ingersoll SB, Sankpal UT, et al. (2011) Tolfenamic acid inhibits ovarian cancer cell growth and decreases the expression of c-Met and survivin through suppressing specificity protein transcription factors. *Gynecol Oncol* 122:163–170. [PubMed: 21496890]
39. Souza PS, Vasconcelos FC, De Souza Reis FR, Nestal De Moraes G, Maia RC. (2011) P-glycoprotein and survivin simultaneously regulate vincristine-induced apoptosis in chronic myeloid leukemia cells. *Int J Oncol* 39:925–933. [PubMed: 21720708]
40. Takahashi T, Honma Y, Miyake T, et al. (2015) Synergistic combination therapy with cotylenin A and vincristine in multiple myeloma models. *Int J Oncol* 46:1801–1809. [PubMed: 25672400]
41. Li F, Ambrosini G, Chu EY, et al. (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 396:580–584. [PubMed: 9859993]
42. Porter AG, Janicke RU. (1999) Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 6:99–104. [PubMed: 10200555]
43. Springer JE, Azbill RD, Knapp PE. (1999) Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury. *Nat Med* 5:943–946. [PubMed: 10426320]
44. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90:405–413. [PubMed: 9267021]
45. Milner AE, Palmer DH, Hodgkin EA, et al. (2002) Induction of apoptosis by chemotherapeutic drugs: the role of FADD in activation of caspase-8 and synergy with death receptor ligands in ovarian carcinoma cells. *Cell Death Differ* 9:287–300. [PubMed: 11859411]
46. Okun I, Balakin KV, Tkachenko SE, Ivachtchenko AV. (2008) Caspase activity modulators as anticancer agents. *Anticancer Agents Med Chem* 8:322–341. [PubMed: 18393791]
47. Wang X, Jin DY, Wong HL, Feng H, Wong YC, Tsao SW. (2003) MAD2-induced sensitization to vincristine is associated with mitotic arrest and Raf/Bcl-2 phosphorylation in nasopharyngeal carcinoma cells. *Oncogene* 22:109–116. [PubMed: 12527913]
48. Yam CH, Fung TK, Poon RY. (2002) Cyclin A in cell cycle control and cancer. *Cell Mol Life Sci* 59:1317–1326. [PubMed: 12363035]
49. Henglein B, Chenivresse X, Wang J, Eick D, Brechot C. (1994) Structure and cell cycle-regulated transcription of the human cyclin A gene. *Proc Natl Acad Sci U S A* 91:5490–5494. [PubMed: 8202514]
50. Eskerod O (1994) Gastrointestinal tolerance studies on tolfenamic acid in humans and animals. *Pharmacol Toxicol* 75 Suppl 2:44–48.

Figure 1: Sp1 mRNA expression in human cancer cell lines and Kaplan-Meier survival curves for Survivin.

(A) Sp1 expression in various human cancer cell lines in Cancer Cell Line Encyclopedia (CCLE) dataset was obtained, analyzed and represented as $\log_2$ 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.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 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 $\mu\text{g/ml}$) 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 $\pm$ SD of triplicate determinations.

Figure 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 $\mu\text{g/ml}$) 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 $\pm$ SD. Statistical analysis performed by one-way ANOVA using GraphPad Prism V6.0.

3A and 3B: All values were significantly different (p value < 0.05) excluding the bars marked with #.

3C: There was no difference between control and treated samples in H9C2 cells.

CON: Control (DMSO); TA 10: Tolfenamic acid 10 $\mu\text{g/ml}$; TA 15: Tolfenamic acid 15 $\mu\text{g/ml}$; VCR0.5: Vincristine 0.5 ng/ml . TA10+VCR 0.5: Tolfenamic acid 15 $\mu\text{g/ml}$ + Vincristine 0.5 ng/ml ; TA15+VCR .5: Tolfenamic acid 15 $\mu\text{g/ml}$ + Vincristine 0.5 ng/ml .

Figure 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 DMSO (control) or TA (10 or 15 $\mu\text{g/ml}$) 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 $\mu\text{g/ml}$; TA 15: Tolfenamic acid 15 $\mu\text{g/ml}$; VCR0.5: Vincristine 0.5 ng/ml . TA10+VCR 0.5: Tolfenamic acid 15 $\mu\text{g/ml}$ + Vincristine 0.5 ng/ml ; TA15+VCR 0.5: Tolfenamic acid 15 $\mu\text{g/ml}$ + Vincristine 0.5 ng/ml .

Figure 5: Combination treatment with TA and VCR induced apoptotic cell population. CHLA-9 and TC-32 cells were treated with DMSO or TA (15 μ g/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 3 independent experiments were used to compare the results among single or combination treatment. The graphs represent mean $\pm$ 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 μ g/ml; VCR0.5: Vincristine 0.5 ng/ml. TA15+VCR 0.5: Tolfenamic acid 15 μ g/ml + Vincristine 0.5 ng/ml.

Figure 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.

Figure 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.