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Combination of clotam and vincristine enhances anti-proliferative effect in medulloblastoma cells

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Abstract

Medulloblastoma (MB) is characterized by highly invasive embryonal neuro-epithelial tumors that metastasize via cerebrospinal fluid. MB is difficult to treat and the chemotherapy is associated with significant toxicities and potential long-term disabilities. Previously, we showed that small molecule, clotam (tolfenamic acid: TA) inhibited MB cell proliferation and tumor growth in mice by targeting, survivin. Overexpression of survivin is associated with aggressiveness and poor prognosis in several cancers, including MB. The aim of this study was to test combination treatment involving Vincristine[®] (VCR), a standard chemotherapeutic drug for MB and TA against MB cells. DAOY and D283 MB cells were treated with 10 µg/ml TA or VCR (DAOY: 2 ng/ml; D283: 1 ng/ml) or combination (TA+VCR). These optimized doses were lower than individual IC₅₀ values. The effect of single or combination treatment on cell viability (CellTiterGlo kit), Combination Index (Chou-Talalay method based on median-drug effect analysis), activation of apoptosis and cell cycle modulation (by flow cytometry using Annexin V and propidium iodide respectively) and the expression of associated markers including survivin (Western immunoblot) were determined. Combination Index showed moderate synergistic cytotoxic effect in both cells. When compared to individual agents, the combination of TA and VCR increased MB cell growth inhibition, induced apoptosis and caused cell cycle (G₂/M phase) arrest. Survivin expression was also decreased by the combination treatment. TA is effective for inducing the anti-proliferative response of VCR in MB cells. MB has four distinct genetic/molecular subgroups. Experiments were conducted with MB cells representing two subgroups (DAOY: SHH group; D283: group 4/3).

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⁷Conflicts of interest:

All authors declare no conflict of interest.

TA-induced inhibition of survivin expression potentially destabilizes mitotic microtubule assembly, sensitizing MB cells and enhancing the efficacy of VCR.

Keywords

Medulloblastoma; Vincristine; Tolfenamic acid; Combination index; Survivin expression

1. Introduction

Medulloblastoma (MB) is an embryonal tumor of the posterior fossa which is located in cerebellum and has a propensity to disseminate throughout the central nervous system (Louis et al., 2016). Each year, 400 to 500 children in the United States are diagnosed with MB, with more frequent occurrences in males than females with a ratio of approximately 2:1 (Polkinghorn and Tarbell, 2007; Gopalakrishnan et al., 2015). Moreover, the overall survival rate in infants is lower (between 30–50%) than in older children as their disease tends to be more aggressive with macroscopic metastatic features (M2/M3) (Gottardo and Gajjar, 2006). Currently, a multimodal approach is applied as the standard treatment strategy for MB. Current standard of care therapy includes gross total resection, craniospinal irradiation and adjuvant chemotherapy (Bourdeaut et al., 2011; Pollack, 2011). Chemotherapy is the preferred option over radiation for children below the age of three years. Vincristine, lomustine, cisplatin, methotrexate and temozolomide are some of the common chemotherapeutic drugs used in treatment regimens of MB (Othman et al., 2014). However, despite good outcomes after treatment many survivors have significant medical problems including neurological, neurocognitive and neuroendocrine sequela that affect their quality of life (Ribi et al., 2005). In this current scenario of chemotherapeutic clinical outcome, it is important to give emphasis on enhancing the efficacy of the present anti-cancer drug treatments while reducing long-term side effects.

Non-steroidal anti-inflammatory drugs (NSAIDs) are known for their anti-inflammatory, anti-pyretic and analgesic properties. Their role as anti-cancer and chemopreventive agents have also been well established via numerous experimental, clinical and epidemiological studies (Rayburn et al., 2009). They promote anti-neoplastic activity by targeting the proliferative, angiogenic and metastatic processes of cancer cells (Liggett et al., 2014; Hilovska et al., 2015). Inhibition of prostaglandin biosynthesis from arachidonic acid by blocking the activities of cyclooxygenase (COX)-1 and COX-2 is the conventional mechanism of NSAIDs (Vane and Botting, 1997; Smith et al., 2000). However, this mechanism has undesirable consequences including gastric bleeding, cardio-toxicity and kidney failure (Thun et al., 2002; Khan and Fraser, 2012). Side effects associated with the COX-dependent mechanism have stimulated research into exploring the various COX-independent enzymatic pathways of NSAIDs (Liggett et al., 2014). Evaluating NSAIDs with lower or negligible toxicities that may modulate specific cellular and molecular targets applicable to cancer therapeutics has become a growing field. One such COX-independent NSAID which has been extensively studied is tolfenamic acid (TA). TA is commercially known as Clotam[®] or Tufnil[®] and is used for treating migraine headaches in Europe and Asia (Vaitkus and Pauza, 2002) and known to cross blood brain barrier (Subaiea et al., 2011).

Compared to other NSAIDs, TA is known to have a low gastro-ulcerogenicity profile (Eslerod, 1994). Interestingly, Our laboratory and others have investigated the anti-tumorigenic activity of TA in pre-clinical models of several different types of malignant cancers, including the pediatric cancers leukemia, neuroblastoma and MB (Abdelrahim et al., 2006; Sankpal et al., 2012; Eslin et al., 2013a; Eslin et al., 2013b; Sutphin et al., 2014). The proposed mechanism for its anti-cancer activity is inhibiting the expression of Specificity protein transcription factor 1 (Sp1) and survivin, an inhibitor of apoptosis protein (IAP) (Basha et al., 2011; Shelake et al., 2017). However, in this investigation, the focus is on survivin due to its association in resistance to chemo-or radiation therapy. Survivin is known for its dual role in inhibiting apoptosis and promoting mitosis. Survivin has a prognostic importance due to its elevated expression in several metastatic cancer types (Ryan et al., 2009). Clinical findings have also associated overexpression of survivin as poor prognosis determinant in MB (Abdel-Aziz et al., 2013). In addition, evidence indicates that TA can regulate the expression of survivin, suggesting it as a viable option in attenuating overexpression of survivin in various tumors (Konduri et al., 2009; Chen et al., 2011). We have previously shown the downregulation of survivin expression by TA in MB cell lines and animal xenograft models lead to tumor growth inhibition (Eslin et al., 2013a).

Understanding the mechanisms by which NSAIDs have an anticancer effect has helped in understanding how to incorporate these medications in the therapy. There are numerous studies, both preclinical and clinical, that have demonstrated the use of such anti-inflammatory and analgesic agents as effective adjuvants for conventional chemotherapies (de Groot et al., 2007). Identifying, which standard of care chemotherapy medications to use in conjunction with anticancer NSAIDs has the potential to show increased efficacy and possibly decreased long-term side effects. Vincristine® (VCR), a commonly used chemotherapeutic agent for MB treatment, is known to induce neurotoxic effects in children such as peripheral neuropathies and seizures (von Bueren et al., 2009). We have undertaken this study to see whether or not the addition of TA with VCR could lead to increased efficacy of VCR in MB. In this study, we used human MB cell lines (DAOY and D283) to investigate the combination effect of TA and VCR to achieve an enhanced anti-tumorigenic effect. We hypothesize that the addition of TA sensitizes MB cells to the chemotherapeutic drug VCR by targeting survivin expression.

2. Methods

2.1. Preparation of Stock Concentrations

TA, VCR and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (St. Louis, MO). Stocks of TA (50 mM dissolved in DMSO) and VCR (1 mg/mL in Dulbecco's phosphate buffered saline (DPBS, Hyclone, Logan, UT)) were prepared for treatment purposes. The stock of VCR was stored at -20°C, whereas, the stock of TA was stored at room temperature.

2.2. Cell Lines and Culture Conditions

Two human derived MB cell lines, DAOY and D283, were procured from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were grown in Eagle's minimal

essential media (EMEM) supplemented with 5% fetal bovine serum (FBS) from HyClone. The culture conditions for cell propagation and various assays listed below were maintained at 37°C with 5% CO₂ in humidified incubator. Deidentified conceptual tissues were obtained from the Birth Defects Research Laboratory at the University of Washington (UW) in Seattle in full accordance with state and federal guidelines (UW Human Subjects Division Institutional Review Board (IRB) # STUDY00000380). The use of deidentified conceptual tissues for the preparation of human neural cell cultures was reviewed and approved by the North Texas Regional IRB (protocol # 2007–121). Primary human astrocytes of non-cancerous origin from three biological donors were isolated and cultivated as described previously (Gardner et al., 2006). The cells were cultured in Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12–1:1) supplemented with 10% FBS (Peak Serum, Fort Collins, CO), and 1% each of penicillin–streptomycin–neomycin (PSN) and amphotericin B, a fungicidal (Sigma).

2.3. Cell Viability Assay

The effect of drug treatment on cell viability was determined using CellTiter-Glo kit (Promega, Madison, WI) as per the manufacturer's instructions. DAOY and D283 cells were seeded in 96-well plates (Lonza, Basel, Switzerland) at a density of 2,500 cells and 4,000 cells per well respectively, suspended in 100 µL media. Cells were treated with DMSO (vehicle control), TA (5, 10, 20, 30 or 50 µg/mL for DAOY and D283) and VCR (1, 2, 5, 10, 20 or 50 ng/mL for DAOY; 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100 or 200 ng/mL for D283), and cell viability was assessed at 48 h post-treatment. Human astrocytes were plated at the density of 50,000 cells per well. Triplicate wells were treated with DMSO (vehicle control) or TA (10 µg/mL) or VCR (1 or 2 ng/mL) or TA+VCR for 48 h. The cell viability assay was repeated in three independent astrocyte cultures (n=3). The treated cells were then incubated with 100 µL of assay reagent in the dark for 20–30 min followed by measuring of luminescence using SYNERGY HT microplate reader (Biotek, Winooski, Vermont). The data obtained was normalized to vehicle control and presented as percentage of viable cells verses drug concentration. Each triplicate measurement was expressed as mean ± standard error of the mean (SEM).

2.4. Combination Index (CI)

The growth inhibitory effect of individual and combination treatment at different doses [TA (10 µg/mL) and VCR (1 or 2 ng/mL)] was measured using cell viability assay as described above. This cell viability data was used to determine the potency (D_m) of the combination based on the growth inhibitor sigmoidal curve represented as m (slope). D_m represents a dose D where half of the cells are alive. To quantitatively determine the nature (synergistic/additive/antagonistic) of the combination effect, CI of TA and VCR was evaluated using Chou-Talalay method based on median-drug effect analysis (logarithmic scale) and the CI value was generated by means of Calcsyn/Compusyn computer software (Biosoft, Cambridge, United Kingdom) using the CI equation. Following median-effect equation (MEE) was used to derive the CI equation: $f_a/f_u = (D/D_m)^m$ - wherein, f_a and f_u are the fraction of cells affected or unaffected by the dose (D), respectively. The evaluation of linear correlation coefficient (r) of the median-effect plot further helped in statistically determining the conformity of the data, which is based on the mass-action law principle. For the current

comparison, the fractional inhibitory values, obtained from the cell viability assay of the individual and combination dose effect, was used for CI calculation based on the non-constant interactive ratio of the two drugs.

2.5. Apoptosis (Annexin-V Staining)

The apoptotic cell population were measured using the Annexin-V-PE (phycoerythrin)/7-AAD (7-amino-actinomycin) apoptosis detection kit (BD Biosciences, San Jose, CA). MB cells were treated with DMSO, TA (10 $\mu\text{g/mL}$), VCR (DAOY – 2 ng/mL ; D283 – 1 ng/mL) or combination. The treated cells were then collected at 24 h and 48 h timepoints to obtain single cell suspension and briefly washed with PBS. These cells were resuspended in 1X binding buffer and further incubated in the dark with Annexin V-PE antibody and 7-AAD for 20 minutes at room temperature. Cells were analyzed using the Beckman Coulter's Cytomics FC500 flow cytometer (Brea, CA), followed by fluorescence compensation using CXP software V2.2 (Beckman Coulter, Inc.) and data analysis using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR). The processed data was then represented as a percentage of (early or late) apoptotic or non-apoptotic cells in the analyzed cell population. The assay was replicated four times and fold increase in apoptotic cell population with respect to the control was calculated for each set. The quadruplet data was shown as mean $\pm$ SEM.

2.6. Caspase 3/7 Activation

The effect of the individual and combination treatment on the activity of the effector caspases was assessed using Caspase-Glo 3/7 kit (Promega) as per the instructions provided by the supplier. The assay set-up, culture conditions and treatment procedures employed were the same as described earlier for the cell viability assay. The treated cells were incubated with 100 μL of assay reagent in the dark for 60 minutes to measure the luminescence values from each well, corresponding to the caspase 3 and 7 activity, using SYNERGY HT microplate reader. The readings obtained were normalized to vehicle control and were presented as fold change in caspase activity. All the treatments were performed in triplicates and the data was represented as mean $\pm$ SEM.

2.7. Protein Extraction and Western Blotting

Cells were treated with DMSO, TA, VCR or combination for 24 and 48 h. Total cellular protein extracts and western blotting was then done using the previously described method (Abdelrahim et al., 2006). Proteins of interest were probed by specific primary antibodies of apoptotic markers, cleaved poly-ADP-ribose polymerase (c-PARP, Cell Signaling Technology, Danvers, MA) and survivin (R&D Systems, Minneapolis, MN), and cell cycle markers cyclin A, cyclin D3 (Cell Signaling Technology), cyclin B1 and cyclin dependent kinases 4/6 (CDK4/6, Santa Cruz Biotechnology, Santa Cruz, CA). The expression of β -actin (Sigma) was used as a loading control.

2.8. Cell Cycle Phase Determination

MB cells were treated with DMSO, TA (10 $\mu\text{g/mL}$), VCR (DAOY – 2 ng/mL ; D283 – 1 ng/mL) or combination. The cells were harvested at 12, 24 and 48 h post-treatment and washed by PBS. Cells were then fixed in cold 70% ethanol overnight at -20°C . Later, cells

were resuspended in propidium iodide (PI) buffer (0.20 µg/mL PI, 20 µg/mL RNase A in PBS) and incubated at room temperature for 20 minutes in the dark. The PI staining was followed by flow cytometry reading using the Beckman Coulter's Cytomics FC500 flow cytometer. The data obtained was analyzed using FlowJo software V8.0 and represented as a percentage of cells in three different phases of cell cycle, G₀/G₁, S and G₂/M. The cell cycle assay was replicated four times. The quadruplet data was shown as mean ± SEM of the fold change values.

2.9. Statistical Analysis:

The data was tested in triplicates and presented as the mean ± SEM using GraphPad Prism 6. IC₅₀ dose response values for TA and VCR at 48 h were evaluated using one-way ANOVA. Two-way ANOVA was used to identify the significant differences among drug-dose combination assays. Post-hoc analysis for pairwise comparison was done using Tukey's honest significant difference (HSD) between different treatment groups and Sidak (as recommended by GraphPad) was used for different treatment-timepoints. The statistical analysis results with *p* value < 0.05 were considered significant.

3. Results

3.1. Growth inhibitory activity of TA and VCR against MB cells:

Cells were treated with DMSO (control), TA (5–50 µg/mL for DAOY and D283) and VCR (1–50 ng/mL for DAOY; 0.1–200 ng/mL for D283), and cell viability was assessed at 48 h post-treatment. For both cell lines, TA and VCR demonstrated a dose-dependent inhibition of cell growth (Figure 1). The IC₅₀ values of the two agents were calculated from the given dose curves. The IC₅₀ values for TA and VCR were 14.06 µg/mL and 4.3 ng/mL in DAOY cells and 13.72 µg/mL and 12.06 ng/mL in D283 cells, respectively. The IC₅₀ values of each drug were used as a baseline to determine the doses for the combination treatment screening experiments; 10 µg/mL of TA (TA10) in both cell lines, 1 ng/mL of VCR (VCR1) in DAOY and 2 ng/mL (VCR2) in D283 (Figure 1).

3.2. Combination of TA and VCR results in increased inhibition of MB cell growth:

Selection of the combination treatment at a given dose was based on the following criteria: (i) the inhibitory effect of the combination should be more than 50%, (ii) whereas the effect of individual treatment must be less than 50%, (iii) we expected to observe a maximum difference of growth inhibition between the combined treatment and respective individual treatments to classify it as an effective dose, and (iv) to choose the lowest possible effective dose tested. Interestingly, the various combination doses that were tested resulted in increased inhibition of MB cell growth in a time- and dose-dependent manner (Figure 2). This decrease in cell growth was comparable to their respective individual treatments and controls.

The growth inhibition of DAOY cells treated with TA10+VCR1 combination was 34.19% (24 h) and 53.04% (48 h), and with TA10+VCR2 was 35.03% (24 h) and 71.74% (48 h). The combination treatments in D283 cells resulted in growth inhibition of 25.81% (24 h) and 66.27% (48 h) for TA10+VCR1, and 33.77% (24 h) and 65.3% (48 h) for TA10+VCR2.

The percent increase in growth inhibition identified at 48 h post-treatment for TA10 and VCR1/2 combination compared to their corresponding single treatments were as follows: in DAOY cells, TA10+VCR1 caused 16.7% and 48.75% increase compared to TA10 and VCR1 alone, and TA10+VCR2 caused 35.4% and 53.71% increase compared to TA10 and VCR2, respectively. In D283 cells, TA10+VCR1 resulted in 33.84% and 43.17% increase compared to TA10 and VCR1, whereas TA10+VCR2 caused 32.87% and 16.86% increase compared to TA10 and VCR2, respectively. Further, the cytotoxic effect of other combinations did not fulfil all the criteria required for treatment selection (Supplementary data Figure S1). Considering these observations, the combination of TA10 with VCR1/2 was selected to further analyze the anti-proliferative effect. Moreover, determination of the CI value (Figure 3A) was used in selecting the doses for each cell line (DAOY: TA 10 µg/mL + VCR 2 ng/mL; D283: TA 10 µg/mL + VCR 1 ng/mL) and demonstrating the moderate synergistic cytotoxic effect of the two drugs on cell proliferation at 48 h (DAOY: CI = 0.774 and D283: CI = 0.727 for the selected doses). The selection of the doses was based on the least CI values obtained for 48 h time-point.

3.3. Non-toxic effect of TA+VCR in normal primary astrocyte cells:

Considering the principal idea of this study of identifying an effective anti-cancer combination dose with reduced peripheral or systemic toxicity, we treated the human derived primary astrocyte cells derived from three donors of non-cancerous origin with our selected doses of TA (10 µg/mL) and VCR (1 or 2 ng/mL) combination for 48 h. The combination treatment did not induce any cytotoxic effect in normal astrocyte cells (Figure 3B), signifying its anti-proliferative effect specific to MB cells.

3.4. TA+VCR induces apoptosis in MB cells:

Apoptotic cell populations were assessed to evaluate the mechanisms involved in anti-proliferative effects induced by the combination treatment of TA10 with VCR2 or VCR1 in DAOY and D283 cells, respectively. The apoptotic cell populations were measured using an Annexin-V-PE/7-AAD kit at 24 and 48 h post-treatment (Figure 4). Annexin-V and 7-AAD positive cells represent late apoptotic cells (LA), whereas, only annexin-V positive cells represent early apoptotic cells (EA). Cells negative to both stains correspond to live cells. The combination treatment caused a significant dose-dependent increase in apoptotic cells. The early and late apoptotic cell populations increased by 2.7-fold and 2-fold at 48 h post-treatment in DAOY and D283 cell lines, respectively (Supplementary data Figure S2). Further, the data illustrates that the percentage of apoptotic and dead cell populations were higher in the combination treatments compared to the respective single treatments. In addition, the combination effect on the activity of key effector caspases was evaluated. The changes observed in caspase 3/7 activity was found to correlate with the cell viability data (Figure 5).

The TA and VCR combination significantly increased the caspase 3/7 activity compared to the individual treatment in a time- and dose-dependent manner. In DAOY cells, approximately 15-fold and 19-fold increase in caspase 3/7 activity was observed at 24 and 48 h, respectively. Alternatively, in D283 cells, the increase in caspase 3/7 activity was 3.6-fold and 9.7-fold at 24 and 48 h, respectively. Moreover, these observations were consistent

with the increase in c-PARP protein expression (Figure 6), which is the downstream target of activated signaling cascade of apoptotic pathway. Increase in c-PARP expression was observed for both cell lines treated with TA and VCR combination at 24 and 48 h post-treatment. These results suggest that induction of apoptosis is one of the crucial mechanisms leading to the enhanced anti-proliferative activity of the combination treatment.

3.5. TA+VCR decreases survivin expression in MB cells:

Survivin is a crucial bio-marker since it is known for its dual role in regulating apoptosis and cell cycle. In addition, our previous pre-clinical studies in several cancer models have identified survivin as a potential therapeutic target of TA. Therefore, we evaluated the expression of survivin by Western blot analysis, in an attempt to understand the anti-proliferative response of the combination treatment. The TA+VCR combination down-regulated the protein expression of survivin in both DAOY and D283 cells (Figure 6). This decrease in survivin expression at 48 h correlates with the growth inhibitory response of TA +VCR treatment.

3.6. TA+VCR causes cell cycle arrest in MB cells:

Apart from induction of apoptosis, cell cycle arrest could also be a potential mechanism causing the decrease in cell viability of TA+VCR treated cells. Therefore, we analyzed the cell cycle phase distribution of both MB cell lines using flow cytometry. The effect of the individual and combination treatments was evaluated at 12, 24 and 48 h post treatment (Supplementary data Figure S3). We observed an increase in the number of cells arrested in G₂/M phase at 48 h (Figure 7A&B). However, a minimal increase in G₀/G₁ and G₂/M phase arrest was observed for the individual treatments with TA and VCR, respectively. It is known that the progression of the cell cycle through its specific checkpoints is primarily regulated by the cyclin-CDK complexes. Any alteration in the threshold of these protein kinases leads to cell cycle arrest (Weinberg, 2013).

To determine the cause of G₂/M arrest due to the combination treatment we evaluated the protein expression of cyclin A, B1, D3 and CDK4/6. We observed that our combination doses resulted in decreased expression of cyclin A, cyclin B1, CDK4 and CDK6 in both cell lines at 48 h, when compared to the control and individual treatments (Figure 7C&D). Likewise, we saw that cyclin D3 expression was downregulated, but only in DAOY cells. These results indicate that the changes in the expression levels of these proteins may contribute towards the cell cycle arrest induced by the TA+VCR treatment.

4. Discussion

VCR is a conventional chemotherapeutic agent used to treat several malignant cancers, including medulloblastoma (Kim et al., 2013). VCR (also known as leurocristine or Oncovin®) is a vinca alkaloid that blocks mitosis by binding to the tubulin protein of the mitotic spindle apparatus (Jordan, 2002). Although VCR currently plays a significant role in the successful treatment of several childhood cancers, the long-term side effects can cause significant issues for quality of life of the survivors (Vazquez et al., 2011). The common side effects caused by VCR are peripheral neuropathy including paresthesia, hyponatremia, and

hair loss (Postma et al., 1993). One possible way to decrease side effects of chemotherapeutic agents is to decrease the total dose used. To address this issue, we have evaluated the use of an adjuvant therapeutic strategy to enhance the efficacy and which may reduce the toxicity. Several small molecule NSAIDs are being studied as adjuvants in cancer therapeutics (Hilovska et al., 2015). Our lab and others have demonstrated TA as a potent anti-cancer agent with a potential to increase the effectiveness of standard therapies (Shelake et al., 2015; Sankpal et al., 2016).

In the present study, we tested the anti-proliferative effect of VCR when combined with TA. We found that TA significantly enhanced the growth inhibitory ability of VCR in MB cells with a moderate synergistic effect. We were able to identify the effective combination dose in MB cell lines from the cell viability based screening assays and selection criterion. This combination synergistically enhanced the anti-proliferative activity in MB cells in a time- and dose-dependent manner. We found that, TA significantly enhances the cell growth inhibition efficiency of VCR in DAOY and D283 cell lines at a drug concentration of less than the IC_{50} value of the individual drug. Even though the combination treatment is potent in specifically targeting cancer cell proliferation, the apparent growth inhibitory effect of the given dose needs to be tested on normal cells as well. However, a normal cell line model representing MB is currently not available. As an alternative, we have used other brain derived normal (non-cancerous) cell lines or primary cultures like astrocytes to test the toxic effect of our combination treatment. The combination of TA and VCR did not induce any cytotoxic effect in non-cancerous astrocyte cells, serving the purpose of this preliminary study. Recently, we demonstrated that TA and VCR combination did not induce toxicity in cardiomyocytes (H9C2 cells) at the concentrations that inhibited Ewing sarcoma cancer cell growth by 70% (Shelake et al., 2019).

Evading apoptosis is one of the hallmarks of cancer and thus is a favored target of therapeutic strategies. In the present study, we establish a link between the anti-proliferative activity of the combination drug treatment with apoptosis initiation. Under standard physiological conditions, the cellular processes of apoptosis are activated via either of the two commonly known pathways, intrinsic pathway and/or extrinsic pathways. The intrinsic and extrinsic pathways ultimately converge towards activation of executioner caspases (3/7) via initiator caspases 9 and 8, respectively. Caspase 3 and 7 cause DNA fragmentation, cleavage of cytoskeletal elements and cleavage of several vital cellular proteins including 113 kDa PARP into fragments of 24 and 89 kDa (Herceg and Wang, 1999; Lavrik et al., 2005). Our results for combination treatment have demonstrated that the upregulation of the proapoptotic marker, c-PARP, correlates with the increased activity of caspase 3/7 in a time- and dose-dependent manner.

In addition, changes to the biochemical features of the outer plasma membrane contribute towards non-inflammatory recognition of the apoptotic cells by phagocytic cells (Maderna and Godson, 2003). This phenomenon is characterized by the expression of the phosphatidylserine (PS) on the outer leaflet of the plasma membrane during the early apoptotic phase and is known to be one of the hallmarks of apoptosis. Due to the high binding affinity of annexin-V for PS, identification of apoptotic cell population has become feasible via biochemical assays. Our flow cytometry analysis has helped in measuring the

percentage of apoptotic cells resulting from the individual or combination treatments of MB cells using annexin-V-PE/7-AAD staining. We observed that the combination treatment of TA+VCR at 48 h caused a significant increase in annexin-V positive cells compared to the control or either agent alone. These results indicate the activation of apoptosis is greater with TA+VCR treatment than with the standard drug VCR alone.

Survivin belongs to the IAP class of gene family and its overexpression is associated with tumor progression, invasiveness and therapeutic resistance leading to poor outcome in several cancers, including MB (Abdel-Aziz et al., 2013). Survivin is identified as a potential therapeutic target due to its dual role as an inhibitor of apoptosis and regulator of mitosis progression (Mita et al., 2008; Ryan et al., 2009). During normal cellular conditions, the regulated expression of survivin is noted during the G₂/M phase as it associates and stabilizes the microtubule filaments of the mitotic spindle apparatus in a precise and saturable reaction governed by microtubule dynamics (Altieri et al., 1998). This mechanism is also responsible for inducing resistance in cancer cells by counteracting the therapeutic outcome of microtubule depolymerizing drugs like vinka alkaloids. However, inhibition of the survivin-microtubule interaction is known to increase the caspase 3 and 7 activity due to the loss of survivin's anti-apoptotic function (Shin et al., 2001). In addition, the resulting destabilization of the alpha- and beta-tubulin polymers arrests the cells at the M phase, which is regulated by the spindle arrest checkpoint (Beardmore et al., 2004). The study by Brun *et.al.* (2015), reported that genetic deletion of survivin expression in tumor cell models causes cell cycle arrest at G₂/M phase (Brun et al., 2015). The observations of this study can be correlated to the above stated facts, wherein the TA+VCR co-treatment substantially induced G₂/M arrest with decreased expression of survivin. However, such enhanced effect on cell cycle arrest was not observed with the individual treatment of VCR due to lack of survivin inhibition. Here, the uninhibited expression of survivin would be counteracting the mechanism of this microtubule destabilizer (Supplementary data S4). Our prior studies have demonstrated the inhibition of survivin by TA in a dose-dependent manner. Hence, based on our overall observations, we are proposing that the adjuvant treatment of TA sensitizes the MB cells towards the reduced dose of VCR by inhibiting the survivin induced microtubule stabilization at M phase.

Cell cycle regulation is a complex process controlled by extrinsic and intrinsic stimuli. For the successful duplication of the cells, cell cycle is guarded by specific checkpoints. These checkpoints ensure the completion of each cell cycle event before it progresses to the next phase. Cumulative expression of cyclins and their associated CDK's regulate the progression of the cell cycle (Pucci et al., 2000). Therefore, down-regulation of these cyclins or CDK's would determine the block of the cell cycle at a certain phase. This study demonstrated that the TA+VCR combination noticeably decreased the expression of CDK4 and CDK6. When complexed with cyclin D, they permit the progression of the cell into S phase. Inhibitors of these CDKs are extensively studied for treating various cancers types by causing arrest at the G₀/G₁ phase (Otto and Sicinski, 2017). Whiteway *et al.* in 2013 have reported the overexpression of CDK6 in MB as a potential therapeutic target to suppress the cell proliferation (Whiteway et al., 2013). Even though our current CDK4/6 and cyclin D protein expression data do not correlate with the increased G₂/M arrest of MB cells, their down-regulation by the TA+VCR co-treatment opens the opportunity for further investigation.

However, the down-regulation of cyclin A and cyclin B1 could be associated with the increased cell population at S and G₂/M phase caused by the combination treatment. Cyclin A/CDK2 complex is predominantly required during S phase to initiate DNA replication, whereas accumulation of cyclin A and cyclin B1 during G₂ phase promotes mitosis when coupled with CDK1 (Yam et al., 2002). The TA+VCR combination treatment demonstrated an increase in the number of cells arrested at G₂/M phase contributing to its anti-proliferative efficacy.

5. Conclusion

In conclusion, this study illustrates that the co-treatment of TA with lower doses of VCR synergistically enhanced the inhibition of MB cell proliferation compared to VCR alone, with a suggestive role of survivin inhibition. While some combinations may work one of the sub-groups of MB, this combination is indiscriminately working against the cells lines representing distinct molecular subgroups (DAOY: SHH group; D283: group 4/3) demonstration broader application. Moreover, the anti-proliferative effect of the combination treatment was accompanied by an increase in apoptosis and obstruction of cell cycle progression determined by their respective markers. This novel combination strategy may provide both an increased efficacy for the treatment of MB, with the possibility of decreasing long-term side effects associated with VCR. The next steps in understanding both efficacy and tolerability will be to evaluate the combination in mouse xenograft models of MB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Dose Selection for Screening of Combination Effect			
TA	5 µg/mL	10 µg/mL	15 µg/mL
VCR	1 ng/mL	2 ng/mL	

Figure 1: Anti-proliferative activity of TA and VCR in MB cells.

(A&B) DAOY and (C&D) D283 cells were treated with DMSO (control) or increasing concentrations of TA (5, 10, 20, 30, 50 µg/mL for DAOY and D283) or VCR (1, 2, 5, 10, 20, 50 ng/mL for DAOY; 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 ng/mL for D283). Cell viability was measured at 48 h post-treatment using CellTiter-Glo kit. Percent viable cells over control were calculated and plotted against log of TA or VCR doses to determine the IC₅₀ values from the respective dose curves. Treatments were done in triplicates and represented as mean ± SEM. The dose curve effect was statistically significant as determined using one-way ANOVA ($p < 0.01$).

Figure 2: Anti-proliferative activity of TA+VCR combination in MB cells.

(A) DAOY and (B) D283 cells were treated with DMSO (control) or TA10 (10 µg/mL) or VCR1/2 (1 or 2 ng/mL) or combination of TA+VCR. Cell viability was measured at 24 h and 48 h post-treatment using CellTiter-Glo kit. Percent viable cells over control were calculated and plotted against each treatment doses. Data represented as mean $\pm$ SEM for each triplicate measurement. The cytotoxic effect of indicated combination treatments with the respective single treatment and control is considered significant as determined via two-

way ANOVA and post-hoc analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ##### $p < 0.0001$).

A

	DAOY		D283	
	24 h	48 h	24 h	48 h
TA10 + VCR1	1.038	0.949	1.007	<u>0.727</u>
TA10 + VCR2	1.217	<u>0.774</u>	0.936	0.868

B

Figure 3: Combination index of TA+VCR and its effect on primary astrocytes.

(A) The table represents the combination index (CI) values for TA10 (10 µg/mL) and VCR1 (1 ng/mL) or VCR2 (2 ng/mL) co-treatments in MB cell lines at 48 h. CI values were calculated using the mean of percentage viable cells measured via cytotoxicity assay. These calculations are based on Chou Talalay's median-drug effect equation. Calcsyn software was employed for this analysis and determination of CI values. (B) Primary normal astrocyte cells were treated with DMSO (control) or TA10 or VCR1 or VCR2 or TA10+VCR1/2. Cell viability was measured at 24 and 48 h post-treatment using CellTiter-Glo kit. Percent viable cells over control were calculated and plotted against each treatment doses. Data represented as mean ± SEM for each triplicate measurement and analyzed by one-way ANOVA.

Figure 4: Apoptotic effect of TA+VCR on MB cells.

(A) DAOY and (B) D283 cells were treated with DMSO (control) or TA10 (10 μ g/mL) or VCR1/2 (DAOY: 2 ng/mL; D283: 1 ng/mL) or both. Apoptotic cell populations were assessed by flow cytometry at 24 and 48 h post-treatment using Annexin-V-PE/7-AAD kit. The X-axis and Y-axis represents PE-Annexin-V positive cells and 7-AAD positive cells, respectively. Data is representative of four independent experiments. (EA – Early Apoptotic cells; LA – Late Apoptotic cells).

Figure 5: Effect of TA+VCR on MB cell viability and effector caspase activity.

(A&B) DAOY and (C&D) D283 cells were treated with DMSO (control) or TA10 (10 $\mu\text{g/mL}$) or VCR1/2 (DAOY: 2 ng/mL ; D283: 1 ng/mL) or both. Cell viability and caspase 3/7 activity were determined at 24 and 48 h post-treatment using CellTiter-Glo and Caspase-Glo-3/7 kits, respectively. The growth inhibitory graph signifies the percentage of viable cells (relative to control) against treatment, whereas caspase activity is presented as fold change with respect to the treatments. Data represented as mean $\pm$ SEM for each triplicate measurement. All groups are significantly different from corresponding controls (* $p < 0.01$) as determined by two-way ANOVA and post-hoc analysis. The effect of combination treatment significantly increases at 48 h compared to the individual treatments as indicated (** $p < 0.001$; *** $p < 0.0001$; #### $p < 0.0001$).

Figure 6: TA+VCR modulates expression of apoptotic markers.

(A) DAOY and (B) D283 cells were treated with DMSO (control) or TA10 (10 μ g/mL) or VCR1/2 (DAOY: 2 ng/mL; D283: 1 ng/mL) or both for 24 or 48 h. Protein extracts were prepared and protein expression of c-PARP and survivin was evaluated by Western blot analysis. β -actin was used as a loading control.

Figure 7: TA+VCR effect on cell cycle phase distribution in MB cells.

(A) DAOY and (B) D283 cells were treated with DMSO (control) or TA10 (10 $\mu\text{g}/\text{mL}$) or VCR1/2 (DAOY: 2 ng/mL ; D283: 1 ng/mL) or both for 12 h. Cell cycle progression was analyzed by flow cytometry after cell fixing and propidium iodide (PI) staining. The X-axis and Y-axis represents PI intensity and cell count, respectively. Data is representative of four independent experiments. (C) DAOY and (D) D283 cells were treated with DMSO (control) or TA10 (10 $\mu\text{g}/\text{mL}$) or VCR1/2 (DAOY: 2 ng/mL ; D283: 1 ng/mL) or both for 24 and 48 h. Cell lysates were collected and used for protein expression evaluation by Western blot

analysis. The protein expression of cyclin A, B1, D3 and CDK 4/6 was evaluated, while β -actin was used as a loading control.

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