Tolfenamic Acid Enhances the Anti-Proliferative Activity of Vincristine in Medulloblastoma Cell Lines

THESIS

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CHAPTER – I

BACKGROUND LITERATURE

INTRODUCTION: MEDULLOBLASTOMA

World Health Organization (WHO) has characterized medulloblastoma (MB) as one of the most malignant, grade IV disease. MB is an embryonal tumor of the posterior fossa which is located in craniospinal region of the skull and has propensity to disseminate throughout the central nervous system (CNS) [1]. Each year, about 400 to 500 children in the United States of America are diagnosed with MB, with more frequent occurrences in males than females with a ratio of about 2:1 [2,3]. Pertaining to clinical findings and histological subtypes, MB at present is stratified either as average-risk or high-risk disease with progression-free survival (PFS) rate of approximately 75% and 60%, respectively [4,5]. Moreover, the overall survival rate in infants is lower (between 30-50%) as their disease tends to be more aggressive with macroscopic metastatic features (M2/M3) [4]. Currently, a multimodal approach is applied as a standard treatment strategy for MB, with improved success rate. This comprises of maximal surgical resection prior to craniospinal irradiation (majorly with the boost to the posterior fossa) and adjuvant chemotherapy [6,7]. Moreover, chemotherapy is the preferred option over radiation for children below the age of three years. Vincristine, lomustine, cisplatin, prednisone, etoposide, methotrexate, cyclophosphamide and temozolomide are some of the common chemotherapeutic drugs used to treat MBs [8]. However, despite the perceptible improvements in treatment strategies, very few survivors are able to live independently as adults [7,9]. There are other therapeutic challenges that still needs to be addressed as patients are exposed to substantial risk of secondary

malignancies or tumor recurrence [10]. Additionally, the adverse effects resulting from the therapy is evident among the long-term survivors as they suffer from treatment associated neurological, neurocognitive and neuroendocrine sequela that affect their quality of life [9]. The adverse effects can be minimized by developing treatments which can specifically target the MB cells and not healthy ones. This treatment strategy demands for identification of specific drivers of MB, like genetic alterations and mutations of prognostic importance. Nevertheless, we first need to understand that the gene alterations in childhood MBs are 10-fold fewer than the adult solid tumors, i.e. most of these mutations in adults are linked with lifestyle and environmental factors, which are not observed in pediatric cancers [11]. Dysregulation of developmental pathways lie at the crux of underlying pediatric MBs [12]. Hence, understanding such molecular pathways underlying the pathogenesis of MB could be helpful in developing less toxic, effective and tumor directed therapies, further opening new possibilities for treatment and disease stratification [3,13].

Molecular Sub-Groups of Medulloblastoma:

The recent studies that focuses on determining the molecular mechanisms involved in MB development has discerned that certain signaling pathways are predominantly activated in the tumor cells. The collective information obtained from such studies, that are based on expression profiles of the protein-coding genes and CGH (comparative genomic hybridization) array, have classified MB into four distinct molecular subgroups, viz., WNT, SHH (representing Wnt and sonic hedgehog pathway, respectively), group 3 (G3), and group 4 (G4). Each of these molecular subgroups have unique demographics and clinical characteristics. Consideration of such heterogeneity is required while designing future pre-clinical studies and clinical trials for optimal therapeutic outcome. In addition, this molecular

classification system is known to have higher prognostic value compared to the classification system based on histological subtypes like classic, desmoplastic, large cell, anaplastic and MB with extensive nodularity [14,15].

The detailed description of molecular classification system based on four MB groups are as follows [16]:

WNT (Wingless) MB: – MB patients of this subtype with *CTNNB1* mutations, monosomy 6 and nuclear Beta-catenin accumulation (Wnt pathway effector molecule) have a good prognosis and excellent outcomes with a 90% survival rate compared to the other sub-types of MB. Wnt MB subtype is seldom seen in infants, with frequent occurrences in grown up children and teenagers. They are known to have classic histology and are rarely metastatic.

SHH (Sonic Hedgehog) MB – They express germ line mutation in *PTCH1* tumor suppressor gene, that encodes a protein, a negative regulator of SHH signaling pathway. Other genes of these pathways that are aberrantly expressed are *SFSU*, *SMO* and *GLI2*, along with amplification of *MYCN* and *YAP1* gene. The altered genes are majorly found in infants and adults, but with distinction in clinical and molecular aspect. The prognostic factor is age dependent with worse outcome of desmoplasia in pediatric cancers. Their histological classification is unique as they include tumors of four main MB variants - classic, large-cell anaplastic, nodular desmoplastic and medulloblastoma with extensive nodularity. Occurrence of metastasis is uncommon in this group.

Group 3 MB – No germ line mutations have been defined for this subgroup and it is restricted to pediatric patients. The G3 MB patient shows higher incidence of metastasis with aggressive behavior and poor prognosis, which is considered as a negative risk factor in MB with approx. 20-30% survival. Amplification of the *MYC* gene in some cases is known to

have a poor outcome. They have frequent large-cell anaplastic histology. The targetable pathways in G3 MB are not well known, however, *NPR3* is proposed as a biomarker for this subgroup.

Group 4 MB – This subgroup is most common and less well understood among all the MB subtypes. This subgroup is found across all age-group, although rarely in infants and has intermediate prognosis with reduced survival rate in the adults. Their histology shows classic variant with frequent metastasis. The proposed marker for this group is *KCNA1* and *FSTL5* was identified as a marker of high-risk G4 patients.

CHEMOTHERAPY

Over the years, there has been remarkable advancement in cancer chemotherapeutics, leading to improved survival rate and delay in disease progression. Chemotherapy is extensively used for treatment of MB and these grade IV tumors are known to respond positively to this therapeutic modality with improved survival rate [17–19].

Nevertheless, of all anticancer drugs available to date are yet not completely effective in improving the cure rate in most of the cancer cases [20]. There are two major issues associated with chemotherapeutic interventions that causes inadequate response to chemotherapeutic drug dosage, inclusive of its associated adverse effects and the acquisition of drug resistance in cancer [21,22].

Adverse Effects of Chemotherapy

Unlike surgical resection and radiation therapy, chemotherapy has a systemic effect over the patient's body. Majority of the conventional chemotherapeutic drugs targets the rapidly dividing cells like cancer cells. However, this mechanism of action of chemotherapeutic

drugs can also affect normal cells that undergoes frequent cell divisions, for example, hematopoietic stem cells, hair follicles, or cells lining the intestine, mouth or reproductive system. The undesired effect of the anti-neoplastic drugs on normal cells causes either long- or short-term adverse effects in cancer patients. Short-term side effects include fatigue, anemia, hair loss, edema, thrombocytopenia, neutropenia, constipation or problems associated with fertility. Peripheral neuropathy is one of the well-studied side effect observed among the MB patients treated with commonly used drugs like vincristine (vinka alkaloids) and cisplatin (platinum agents) [23]. The side effects experienced by cancer patients are dependent on factors like the drug dose, duration of the treatment, the general health of the patient and the mode of administration of the drug. Although, the overall survival rate or PFS has increased with the application of current rigorous therapeutic regimens, the survivor studies has helped in realizing the long-term treatment associated adverse effects that persist years after the completion of chemotherapy [24]. Studies have also reported that survivors of pediatric MBs suffer from treatment induced cognitive and neurologic health impairment affecting their quality of life [25,26].

Chemoresistance

The development of chemoresistance is one of the major obstacle for successful chemotherapy in treating cancer. Chemoresistance occurs by various mechanisms which can reduce the effectiveness of the drug [27]. It is considered as the prime cause of relapse in cancer. Many cases of tumor recurrence have been noted even after initial success in reduction of primary or malignant tumors post treatment [20]. In certain cases, the failure in treatment is majorly associated with the tumor cell resistance than the pharmacokinetic problems of the cytotoxic agents [21,22]. These observations are not limited to adult

cancers but also eminent in childhood cancers [28,29]. Hence, it is very important to understand and elucidate the causes of chemotherapeutic failure that are prevalent even with advancement in novel and sophisticated treatments.

Many different pharmacologic and/or cellular factors contribute to clinical manifestations of drug resistance phenotypes. Factors that prevent an adequate degree of drug exposure and concentration to tumor cells are considered as pharmacological factors. This is due to inefficient doses, delivery methods and drug metabolism that can be altered in chemoresistance mechanism. These limitations can be addressed by providing high dosage of anticancer drugs, but cannot be applied in clinical maneuvers. In addition, there are other cellular mechanisms that can influence drug sensitivity and metabolism within the tumor cells, which includes drug modification leading to activation or inactivation, altered target enzymes and rapid DNA repair mechanism. These factors reduce the ability of tumor cell to undergo apoptosis, which is the major determinant of drug sensitivity [21,30,31].

NOVEL THERAPEUTIC STRATEGIES

Advancements in the field of chemotherapy has been instrumental in treating MB and a majority of other cancer types, but are associated with treatment induced toxic effects. Therefore, in this current scenario of chemotherapeutic clinical outcome, it is important to give more emphasis on enhancing the efficacy of the present anticancer drug treatments with improved molecular profiling, screening methodology and targeted drug delivery technology, rather than focusing on new drug discoveries. This strategy will allow to develop patient-tailored or personalized therapies that would be influenced by molecular based risk stratification system [27,32].

Conceivably, there are three strategies by which we can address the drawbacks of chemotherapy. Firstly, our primary aim should include alteration of chemotherapeutic regimen so as to reduce the side effects associated with conventional anti-neoplastic agents and increase their therapeutic efficacy. The subsequent approach would be to sensitize the cancer cells towards chemotherapeutic agents by targeting specific molecular mechanism of prognostic importance. For example, inhibition of apoptosis is one of the altered mechanism essential for tumor growth and progression [33]. The most extensively studied molecular markers associated with apoptosis inhibition belong to the IAP (inhibitor of apoptosis proteins) family of genes. Survivin [also known as Baculoviral-IAP-Repeat-Containing 5 (BIRC5)], the IAP family member, is the potential therapeutic target for sensitizing cancer cells, as it is known to be upregulated in several cancers [34,35]. Increased expression of survivin is correlated as negative prognostic marker in pediatric MBs that influences overall clinical outcome and morphology of the tumor, and hence is the primary target of therapeutic strategy tested in the present study [36,37]. Such sensitizing effect could be achieved by using the strategy of combination therapy. This approach considers the complexities of cancer development, wherein multiple pathways are altered possibly resulting in developing resistance to treatment regimens involving single agents [38]. Targeting such multiple pathways will be beneficial in achieving enhanced therapeutic efficacy in complete elimination of tumor and improving patients quality of life [39]. Combination therapeutic strategy is the central idea of this study for MB and will be discussed further in chapter II.

ANTI-TUMORIGENIC PROPERTIES OF TOLFENAMIC ACID

Non-steroidal anti-inflammatory drugs (NSAIDs) are known for their anti-inflammatory, antipyretic and analgesic properties. Their role as anti-cancer and chemopreventive agents have also been well established via numerous experimental, clinical and epidemiological studies [40]. They promote anti-neoplastic activity by targeting the proliferative, angiogenic and metastatic processes of cancer cells. Although, both cyclooxygenase (COX)-dependent and -independent pathways are known to be involved in anti-tumorigenic responses, the elucidation of precise mechanism is still under investigation [41,42]. Inhibition of prostaglandins (PG) biosynthesis from arachidonic acid (AA) by blocking the activities of COX-1 and COX-2 is the conventional mechanism of NSAIDs [43,44]. However, this mechanism has undesirable consequences including gastric bleeding, cardio-toxicity and kidney failure [45–47]. Side effects associated with the COX-dependent mechanism has stimulated research into exploring the various promising COX-independent enzymatic pathways of NSAIDs [41]. This has extended the scope for evolving novel and better drugs with lower or negligible toxicities and modulating specific cellular and molecular targets applicable to cancer therapeutics.

One of such extensively studied COX-independent NSAID is tolfenamic acid (TA). TA is commercially known as Clotam or Tufnil and it is used as a generic medicine for treating migraine headaches in Europe and Asia [48,49]. Its chemical name is 2-([3-Chloro-2-methylphenyl]amino)benzoic acid. Compared to other NSAIDs, TA is known to have a low gastro-ulcerogenicity profile [50]. Our laboratory and others have investigated the anti-tumorigenic activity of TA in pre-clinical models of several different types of malignant cancers, including pediatric cancers like leukemia, neuroblastoma and MB [51–58]. In

support of this potent anti-cancer characteristic of TA, the cellular and organismal toxicity in pre-clinical *in vivo* tumor models was also evaluated [59]. The results of this study demonstrated the selectivity of TA in targeting specific mechanisms of malignant cell growth without causing apparent toxicity at effective anti-tumor doses. The *in vitro* drug response studies of TA show that it induces apoptosis and cell cycle arrest in cancer cells [60]. The tumor growth inhibitory response of TA has also been validated in mouse xenograft models. The primary known target linking its anticancer activity is modulation of survivin expression [61,62]. Survivin is known for its dual role in inhibiting apoptosis and promoting mitosis [63]. Survivin has prognostic importance due to its elevated expression in several metastatic cancer types [64,65]. Clinical findings have also associated with the overexpression of survivin as a poor prognostic determinant in MB [66]. In addition, evidence from other studies indicates the regulation of survivin expression by TA, suggesting it as a viable option in attenuating survivin for positive therapeutic response [67,68] change reference. Likewise, the downregulation of survivin expression has also been validated by our previous *in vitro* and *in vivo* studies with MB cell and animal xenograft models tested for TA-induced tumor growth inhibition [51].

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CHAPTER – II

Research Strategy and Approach

SIGNIFICANCE OF THE STUDY: USING COMBINATION THERAPY

Using NSAIDs (non-steroidal anti-inflammatory drugs), as monotherapy for cancer is not completely effective. Therefore, there are numerous pre-clinical and clinical studies demonstrating the use of such anti-inflammatory and analgesic agents as effective adjuvants for conventional therapies [1]. Considering the complexities of cancer development and its response to treatment, combination therapy is normally administered in current clinical practices using the standard therapeutic approaches [2,3]. There is also evidence illustrating the potential of NSAIDs in inhibiting or preventing the dose-limiting toxicities of commonly used anti-cancer agents [4]. The precise mechanism contributing towards these effects are still under investigation in different cancer models. However, researchers have proposed three primary mechanisms of action following co-administration or pre-treatment of these agents [5]. This includes protection of normal tissue environment from the damaging effects of anti-neoplastic agents along with increasing their maximum tolerated dose. Secondly, the signified combination therapy also causes alterations in pharmacokinetics or metabolism of these conventional chemo-drugs, further leading to reduction in associated systemic toxicities. Lastly, beyond the chemo-protective capability, NSAIDs may also sensitize cancer cells towards the standard chemotherapeutic agents leading to either synergistic or additive effects. These adjuvant therapies are known to enhance the overall efficacy of the anti-tumor drugs together with improving patient's quality of life.

The chemo-sensitizing properties of TA (tolfenamic acid) has been investigated previously by our laboratory. The response of pancreatic cancer cells and tumor to radiation therapy positively increased by the suppressive action of TA on survivin expression [6]. Likewise, a combination study in colon cancer cells illustrated that TA may enhance the growth inhibitory efficacy of curcumin by significantly upregulating apoptosis and Reactive oxygen species (ROS) activity compared to single/individual treatments [7]. The data were further supported by the decreased expression of specificity protein-1 (Sp1), survivin and NFκB (nuclear factor kappa B) translocation to the nucleus. Similar enhanced anti-proliferative responses was observed for TA combination with 13 *cis*-retinoic acid in high-risk neuroblastoma cells [8]. Studies from other laboratories have also demonstrated the synergistic dose-dependent anti-tumor activity of Mithramycin A and TA combination in pancreatic cancer model utilizing nontoxic doses [9]. However, the effective therapeutic dose of single treatment was directly linked with systemic side effects.

APPROACH FOR CURRENT STUDY

Although remarkable advancements in cancer therapeutics have been achieved, the current standard treatment regimen for medulloblastoma (MB) is still aggressive and associated with serious treatment induced morbidity and debilitating toxicity. In clinical practices, the two key factors that influence the efficacy of anti-neoplastic agents are toxicity/side effects and chemoresistance. These factors can limit the response of therapeutic dose of the agent and curtail patient's quality of life. Therefore, it is of prime importance to investigate novel anti-cancer drugs and therapeutic approaches that would enhance the efficacy of current standard care and improve patient quality of life by reducing side effects.

Our goal was to determine if the discovery of a sensitizing agent, such as small molecule (NSAID), could enhance the efficacy of the chemotherapeutic drugs and minimize the dose dependent side effects? Our previous studies on TA that demonstrates its anti-cancer properties and lower toxic capabilities, further bolsters the idea of using TA as an ideal adjuvant with chemotherapeutic drugs. Since survivin is associated with poor prognosis in MB and a potential target of TA, inhibiting survivin expression is the probable targeted mechanism of TA by inducing its sensitizing effect in MB cells. Hence, this novel drug combination could potentially facilitate the reduction of required standard chemotherapeutic dose(s). Results obtained from this preliminary study could prove to be beneficial in designing less aggressive treatment modalities for MB, by minimizing the associated deleterious side effects.

HYPOTHESIS

Our primary goal is to sensitize MB cells towards the current standard chemotherapeutic drugs leading to reduction in the treatment dose and the toxicities associated with it. This could be achieved by specifically targeting markers associated with tumor progression, aggressiveness and chemoresistance like survivin. Studies have shown survivin to be overexpressed in MB cells with both diagnostic and prognostic importance [10]. *Thus, we hypothesize that –* ***Addition of tolfenamic acid (TA) sensitizes medulloblastoma cells to the chemotherapeutic drug(s) by targeting survivin expression.*** The foundation of this theory is based on our previously published data showing that TA increases the efficacy of anti-neoplastic agents in neuroblastoma and ovarian cancer cells via down-regulation of survivin expression [8,11]. The hypothesis will be tested as depicted in following aims:

SPECIFIC AIM 1: TO identify the effective combination using TA and chemotherapeutic agents for inhibiting MB cell growth.

- To calculate the IC_{50} values of TA and two chemotherapeutic agents, Vincristine and Cisplatin in MB cell lines.
- To determine an effective synergistic combination treatment (TA + Chemo-agent) for increased anti-proliferative activity in MB cells.

SPECIFIC AIM 2: To evaluate treatment with combined effect of selected TA and chemotherapeutic agent doses on apoptosis and cell cycle phase distribution in MB cells.

- To measure the apoptotic cell population via Annexin-V staining.
- To determine the activity of apoptotic effectors, caspase 3/7.
- To assess the expression of survivin protein.
- To analyze the cell cycle phase distribution.
- To evaluate the protein level expression of apoptotic and cell cycle markers.

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CHAPTER – III

To Identify the Effective Combination Using Tolfenamic Acid and Chemotherapeutic Agents for Inhibiting Medulloblastoma Cell Growth

INTRODUCTION

Chemotherapy is widely used as a part of multimodality treatment regimen for metastatic medulloblastoma (MB) [1]. Vincristine (VCR) and cisplatin (Cis), the commonly used chemotherapeutic agents for MB treatment, are known to induce neurotoxic effects in children such as peripheral neuropathy or ototoxicity, respectively [2–4]. We suggest that combination therapy with small molecule adjuvants like NSAIDs (non-steroidal anti-inflammatory drugs) may lead to increased drug efficacy of anti-cancer agents, by reducing their dose-limiting toxicities. In our previous dose response study with MB (*in vitro* and *in vivo* disease models), the effective anti-proliferative response of TA (tolfenamic acid) was observed at a dose of 20 µg/mL [5]. Since we are proposing to test TA along with chemotherapeutic agent, it is important to optimize the minimal responsive dose of TA that could be beneficial in enhancing the anti-neoplastic efficiency of chemotherapeutic drug by lowering their dosage.

To serve the purpose of this objective, we calculated the IC_{50} (inhibitory concentration at 50% cell viability) values of TA and two standard chemotherapeutic drugs VCR and Cis from their dose response curve to set a reference point for deciding the effective growth inhibitory combination doses (TA + Chemo-agent). The selected combination doses were

further quantitatively analyzed to measure the synergism in their growth inhibitory dose-response. Here we employed the most widely used method of median-drug effect analysis to evaluate the effectiveness of the combination. This method uses the data obtained from cytotoxicity assay of both the individual drugs and their combination to calculate the combination index (CI) value based on their sigmoidal shaped dose response curve [5]. The median-effect equation (MEE), that derives the CI theorem, defines a unified theory which holds the potency of the drug to explain the common link between single and multiple entities, and their order of dynamics namely first and higher. The theory explains quite distinctively the quantitative definitions of CI, and whether or not combination of drugs results in additive (CI = 1), synergistic (CI < 1) or antagonistic (CI > 1) effects. The potency (D_m) and dose effect curve (m) are different for each drug. If we know the values of m and D_m for each drug, the resulting CI can be easily calculated [7].

MATERIALS AND METHODS

Cell Lines, Reagents and Cell Culture:

MB cell lines, DAOY and D283 of human origin were procured from American Type Culture Collection (ATCC, Manassas, VA). DAOY cells are adherent with polygonal morphology, whereas D283 are epithelial cells that are mixed in nature consisting of both adherent and suspension cells. Both the cells were grown in Eagle's Minimal Essential Media (EMEM) supplemented with 5% Fetal Bovine Serum (FBS). The culture conditions for cell propagation and various assays used in this study were maintained at 37^{o}C with 5% CO_2 in humidified incubator.

The primary human astrocyte cell culture (number 2733, 127th day, female donor) of non-cancerous origin was obtained from Dr. Ghorpade's Laboratory at University of North Texas Health Science Center (UNTHSC, Fort Worth, TX). Procedures involving Isolation, cultivation, activation and propagation of these cells were performed as described previously by Gardner *et. al*., [8]. The primary cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 - 1:1) supplemented with 10% FBS from Peak Serum (Fort Collins, CO), and 1% each of PSN (Penicillin – Streptomycin – Neomycin) and Amphotericin B (Fungicidal) from Sigma Chemical Co. (St. Louis, MO).

EMEM, FBS, trypsin (1X, 0.25%) and d-PBS (Dulbecco's phosphate-buffered saline) were purchased from HyClone (Logan, UT). TA, VCR, Cis and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). CellTiter-Glo kit was bought from Promega (Madison, WI). Stocks of TA [50mM dissolved in DMSO], VCR [1mg/mL in d-PBS] and Cis [3.33 mM in d-PBS] were prepared for treatment purposes. The stocks of VCR and Cis were stored at -20 ^{o}C, whereas, the stock of TA was stored at room temperature. These stocks were further diluted in media to be used at the indicated concentrations for in vitro assays. For each assay, cell treatments were compared to cells treated with DMSO as vehicle control. The amount of DMSO used is equivalent to the amount of TA used for dilutions.

Cell Viability Assay:

The treatment effect (as single or combination treatment) on cell growth was assessed via luminescence based cell viability assay. Cell viability was determined using CellTiter-Glo kit (Promega, Madison, WI) as per the manufacturer's instructions. The measure of cellular ATP (adenosine triphosphate) levels indicated the number of live cells and was directly proportional to the intensity of the luminescence generated [9]. Briefly, DAOY and D283

cells were seeded in a 96-well, white-walled, clear-bottom plates (Fisher) (Lonza, Basel, Switzerland) at a density of 2,500 cells and 4,000 cells per well respectively, suspended in 100uL media [10]. Normal astrocyte cells were plated at the density of 50,000 cells per well [8]. After 24-hour incubation in a cell culture incubator, the cells were treated with DMSO as a vehicle control or with the specified concentrations of TA, VCR or Cis individually and in combination. Each treatment was performed in triplicates, followed by incubation for either 24- or 48-hours. The treated cells were then incubated with 100µL of assay reagent in dark for 20-30 minutes followed by luminescence measurement using SYNERGY HT microplate reader (Biotek). The data obtained was normalized to vehicle control and represented as percentage of viable cells versus drug concentration.

Statistical Analysis:

Cell viability and IC_{50} values of the drugs were statistically analyzed using GraphPad Prism 6 software. Each triplicate measurement was expressed as mean ± SEM (standard error of the mean). The statistical significance of the dose response values for TA, VCR and Cis at 48 hours was evaluated by using one-way ANOVA. Two-way ANOVA was used for combination studies. Pairwise comparison was done using Tukey's multiple comparison test of sample mean, and Sidak's multiple comparison test was used for different treatment timepoints. The statistical analysis results with p value < 0.05 were considered significant.

Combination Index (CI):

The growth inhibitory effect of individual and combination treatment using indicated doses of TA, VCR, Cis 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 Calcusyn/Compusyn computer software using the CI equation [6,7]. 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. (Source: CalcuSyn manual, Biosoft, 2006).

RESULTS

Growth Inhibitory Effect of Tolfenamic Acid, Vincristine and Cisplatin in Medulloblastoma Cell Lines:

Our goal was to derive a dose of the given anti-neoplastic drugs that would inhibit less than 50% cell growth of the MB cells with individual treatment, but demonstrate an enhanced synergistic growth inhibitory response when used in combination with TA. Hence, we assessed the cytotoxic response of TA and two chemotherapeutic agents, VCR and Cis, on proliferation of DAOY and D283 cells by luminescent cell viability assay using CellTiter-Glo Kit as per prior description. The cells were treated with the increasing concentrations of the given drugs: TA (5, 10, 20, 30, 50 µg/mL), 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) and Cis (1, 2, 5, 7.5, 10, 20 µM for DAOY; 0.5, 1, 2,

5, 10, 20 µM for D283), and cell viability was assessed at 48-hour post-treatment. TA, VCR and Cis demonstrated dose-dependent inhibition of cell growth (Figure 3.1). The IC_{50} values of the three anti-cancer agents was evaluated for each MB cell line from the given dose curves. The IC_{50} values calculated for TA, VCR and Cis at 48-hours post-treatment were 14.06 µg/mL, 4.3 ng/mL, 1.69 µM in DAOY cells and 13.72 µg/mL, 12.06 ng/mL, 3.05 µM in D283 cells, respectively.

The IC_{50} values of each drug was used as a baseline to determine the following doses for combination treatment screening experiment:

	Drug Doses		
Tolfenamic Acid (TA)	5 µg/mL	10 µg/mL	15 µg/mL
Vincristine (VCR)	1 ng/mL	2 ng/mL	
Cisplatin (Cis)	1 µM	2 µM	

Combination of Tolfenamic Acid and Vincristine Results in Increased Inhibition of Medulloblastoma Cell Growth:

To serve the purpose of this study of identifying an effective combination dose, we employed primary screening experiments to evaluate the cytotoxic response of TA and chemo-agent co-treatment at various doses. An increase in anti-proliferative activity of the combination treatments (TA+VCR or TA+Cis) compared to their respective individual treatment was evaluated at 24- and 48- hour post-treatment (Table 3.1 and 3.2; Figure 3.2). Interestingly, the various combination doses that were tested resulted in increased inhibition of MB cell growth in time- and dose-dependent manner. However, our selection of the combination treatment at a given dose was based on several criteria as follows: (i)

inhibitory effect of combination should be more than 50%, (ii) whereas 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 effective dose, (iv) to choose the lowest possible effective dose tested, and (v) the treatment effect of combination must be statistically significant compared to the control (untreated cells) and individual treatments.

Based on these norms, we observed that the co-treatment of TA10 (10µg/mL) and VCR1/2 (1 or 2 ng/mL) significantly decreased the cell viability of both DAOY and D283 cell lines (Figure 3.2 C & D). This decrease in cell growth was comparable to their respective individual treatment and control. The growth inhibition of DAOY cells treated with TA10+VCR1 combination was 34.19% (24h) and 53.04% (48h), and with TA10+VCR2 was 35.03% (24h) and 71.74% (48h). Identical combination treatments in D283 cells resulted in growth inhibition of 25.81% (24h) and 66.27% (48h) for TA10+VCR1, and 33.77% (24h) and 65.3% (48h) for TA10+VCR2. In contrast, the growth inhibitory effect of TA10, VCR1 and VCR2 without any combination were relatively less, i.e., 25.95%, 4.39%, 14.77% (24h) and 36.34%, 4.29%, 18.03% (48h) for DAOY cells, and 17.64%, 7.05%, 21.25% (24h) and 32.43%, 23.1%, 48.44% (48h) for D283 cells, respectively. The percent increase in growth inhibition identified at 48-hour post-treatment for TA10 and VCR1/2 combination compared to their corresponding single treatment were as follows: in DAOY cells, TA10+VCR1 caused 16.7% and 48.75% increase than TA10 and VCR1 alone, and TA10+VCR2 caused 35.4% and 53.71% increase than TA10 and VCR2, respectively; In D283 cells, TA10+VCR1 resulted in 33.84% and 43.17% increase than TA10 and VCR1, whereas TA10+VCR2 caused 32.87% and 16.86% increase than TA10 and VCR2, respectively. However, in case of combination treatment of TA10 (10µg/mL) with Cis1/2 (1 or 2 µM), a comparable decrease in cell viability with respect

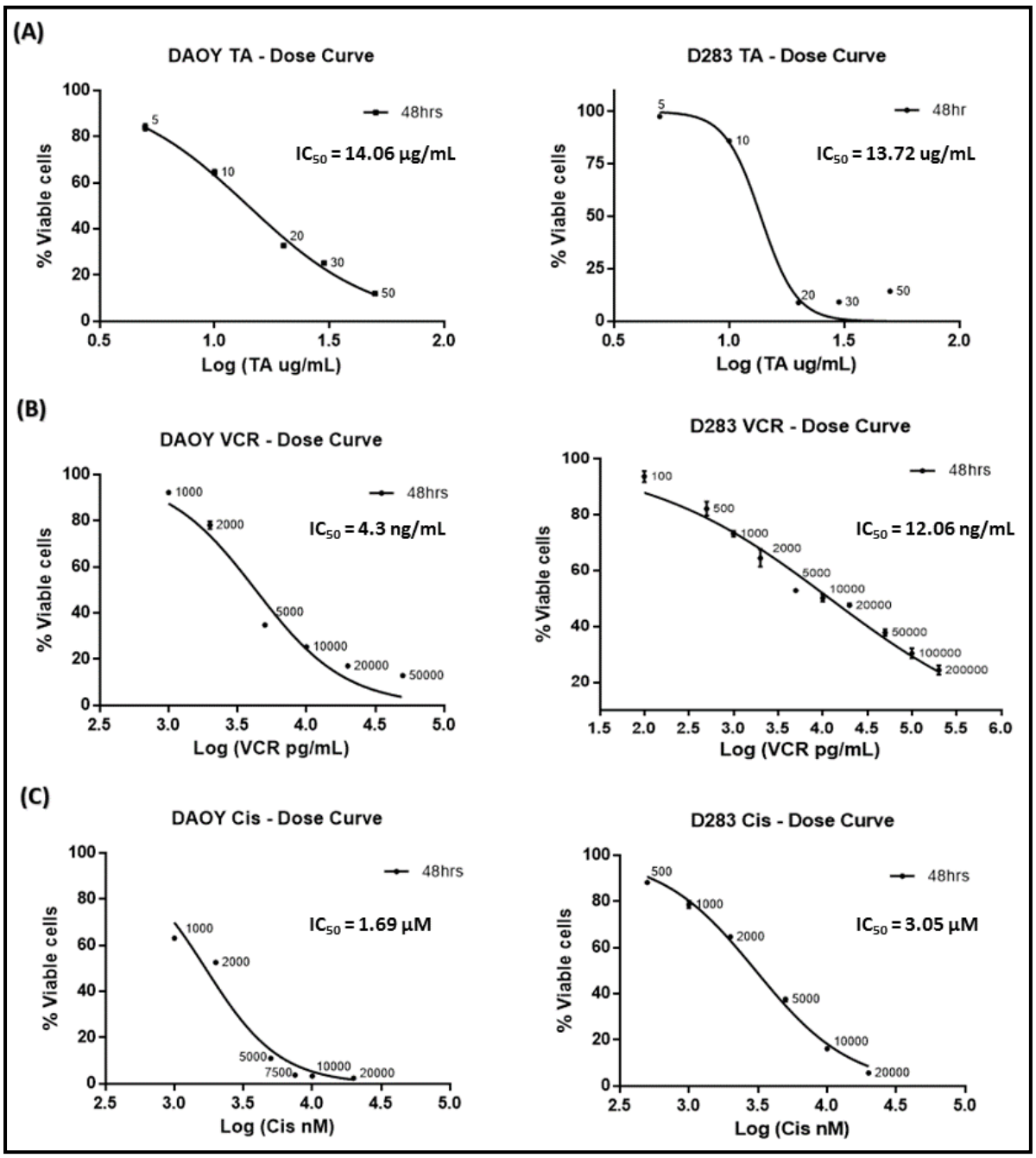
to the stated criteria was observed only in D283 cells and not in DAOY cells. Further, the cytotoxic effect of other combinations did not fulfil all the criteria required for treatment selection like, the co-treatment of VCR or Cis doses with 5 or 15 µg/mL dose of TA was observed to be less effective with respect to their individual treatments. Considering these observations, the combination of TA10 with VCR1/2 was selected (based on the readings at 48-hours) to further analyze its anti-proliferative effect.

Moreover, determination of CI value (Table 3.3) was helpful in selecting the doses for each cell line (DAOY: TA 10µg/mL + VCR 2ng/mL; D283: TA 10µg/mL + VCR 1ng/mL) and demonstrating the synergistic cytotoxic effect of the two drugs on cell proliferation at 48 hours (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-hour time-point.

Non-Toxic Effect of Tolfenamic Acid and Vincristine Combination 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 cell of non-cancerous origin (number 2733, 127th day, female donor) with our selected doses of TA (10µg/mL) and VCR (1 or 2 ng/mL) combination for 24- and 48-hours. The cell viability was measured using luminescent assay as describe in methods section. As shown in Figure 3.3, we observed that the combination treatment did not induce any cytotoxic effect in normal astrocyte cells, signifying its anti-proliferative effect specific to MB cells.

Figure 3.1: **Dose response effect of tolfenamic acid (TA), vincristine (VCR) and cisplatin (Cis) on viability of medulloblastoma cell lines.**



DAOY and D283 were treated with DMSO (vehicle control) or indicated concentrations of **(A)** TA (µg/mL), **(B)** VCR (pg/mL) and **(C)** Cis (nM). Cell viability was measured at 48-hour post-

treatment using CellTiter-Glo kit (Promega). Percent viable cells over control were calculated and plotted against log of TA, VCR or Cis concentrations. The values on the sigmoidal curve indicates the actual concentration of the respective drug doses that were tested. Data represents mean ± SEM of three independent observations, and was used to calculate the IC_{50} value signified for each drug. The dose curve effect is found to be statistically significant as determined using One-way ANOVA (p<0.0001).

Table 3.1: **Time and dose dependent effect of different combination doses of tolfenamic acid with vincristine on viability of medulloblastoma cell lines.**

	Drugs			DAOY		D283	
	DMSO	TA	VCR	24h	48h	24h	48h
		µg/mL	ng/mL	% Viable Cells			
Con	+	-	-	100	100	100	100
VCR1	+	-	1	95.61	95.71	92.95	76.90
VCR2	+	-	2	85.23	81.97	78.75	51.56
TA5	-	5	-	88.30	83.32	93.66	89.94
TA5+VCR1	-	5	1	82.68	76.02	84.57	51.75
TA5+VCR2	-	5	2	78.17	58.09	76.56	46.55
TA10	-	10	-	74.05	63.66	82.36	67.57
TA10+VCR1	-	10	1	65.81	46.96	74.19	33.73
TA10+VCR2	-	10	2	64.97	28.26	66.23	34.70
TA15	-	15	-	56.76	42.67	68.63	36.42
TA15+VCR1	-	15	1	47.60	27.34	58.96	19.60
TA15+VCR2	-	15	2	50.69	20.41	53.20	17.71

DAOY and D283 were treated with either DMSO (vehicle control) or TA (5 or 10 or 15 µg/mL) or VCR (1 or 2 ng/mL) or combination of TA+VCR. Cell viability was measured at 24- and 48-hours post-treatment using CellTiter-Glo kit (Promega). Percent viable cells over control were calculated for each treatment doses. The table represents the percentage mean of three independent observations.

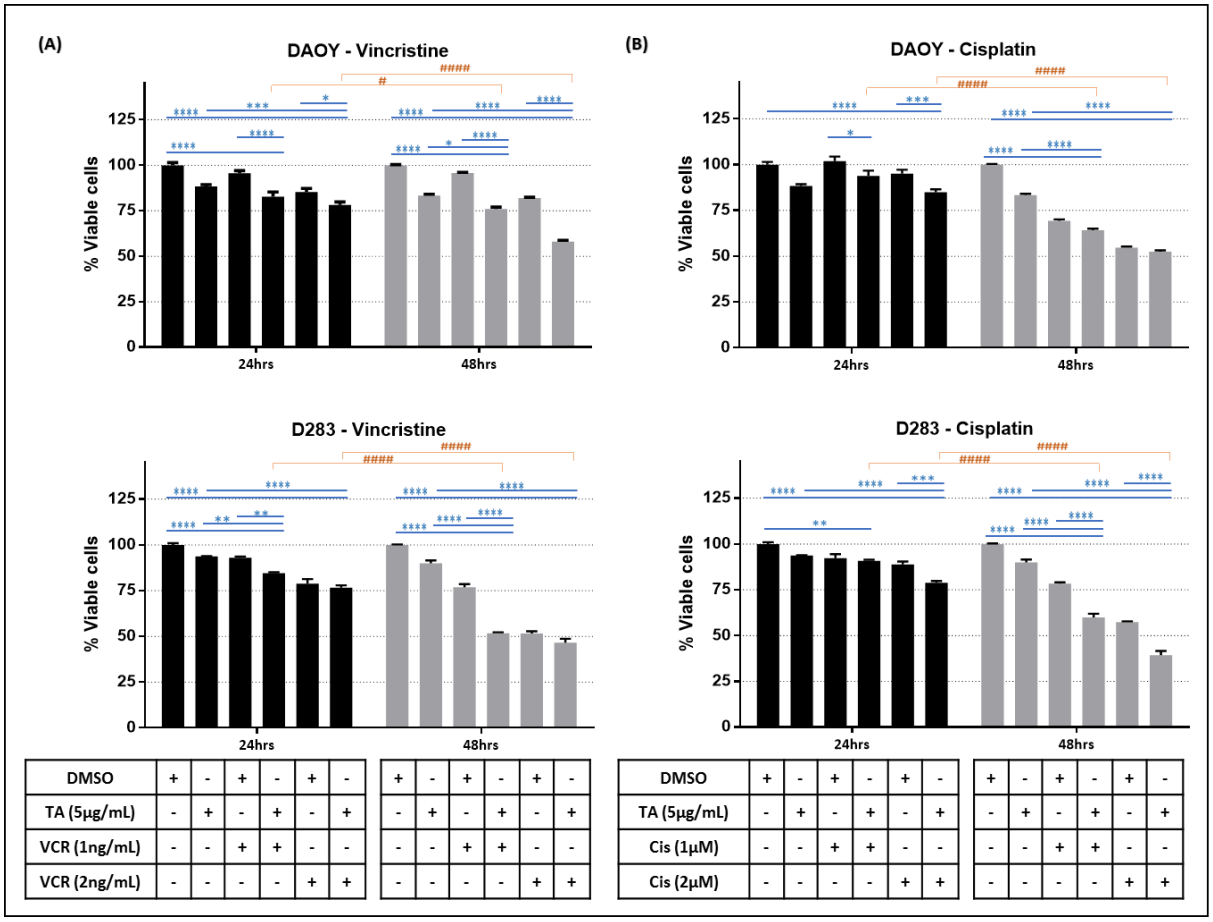
Table 3.2: **Effect of different combination doses of tolfenamic acid with cisplatin on viability of medulloblastoma cell lines.**

	Drugs			DAOY		D283	
	DMSO	TA	Cis	24h	48h	24h	48h
		µg/mL	µM	% Viable Cells			
Con	+	-	-	100	100	100	100
Cis1	+	-	1	101.88	69.26	92.18	78.30
Cis2	+	-	2	95.06	54.74	88.84	57.34
TA5	-	5	-	88.30	83.32	93.66	89.94
TA5+Cis1	-	5	1	93.90	64.15	90.84	59.89
TA5+Cis2	-	5	2	85.02	52.45	78.85	39.21
TA10	-	10	-	74.05	63.66	82.36	67.57
TA10+Cis1	-	10	1	70.81	55.12	78.07	32.60
TA10+Cis2	-	10	2	71.93	46.13	65.59	20.78
TA15	-	15	-	56.76	42.67	68.63	36.42
TA15+Cis1	-	15	1	59.11	42.15	56.30	14.92
TA15+Cis2	-	15	2	57.80	36.85	47.22	12.08

DAOY and D283 were treated with either DMSO (vehicle control) or TA (5 or 10 or 15 µg/mL) or Cis (1 or 2 µM) or combination of TA+Cis. Cell viability was measured at 24- and 48-hours post-treatment using CellTiter-Glo kit (Promega). Percent viable cells over control were calculated for each treatment doses. The table represents the percentage mean of three independent observations.

Figure 3.2: **Combination dose effect of tolfenamic acid (TA) with vincristine (VCR) or cisplatin (Cis) on viability of medulloblastoma cell lines.**

(A, B) Combination of TA (5 µg/mL) with VCR (1 or 2 ng/mL) and Cis (1 or 2 µM)



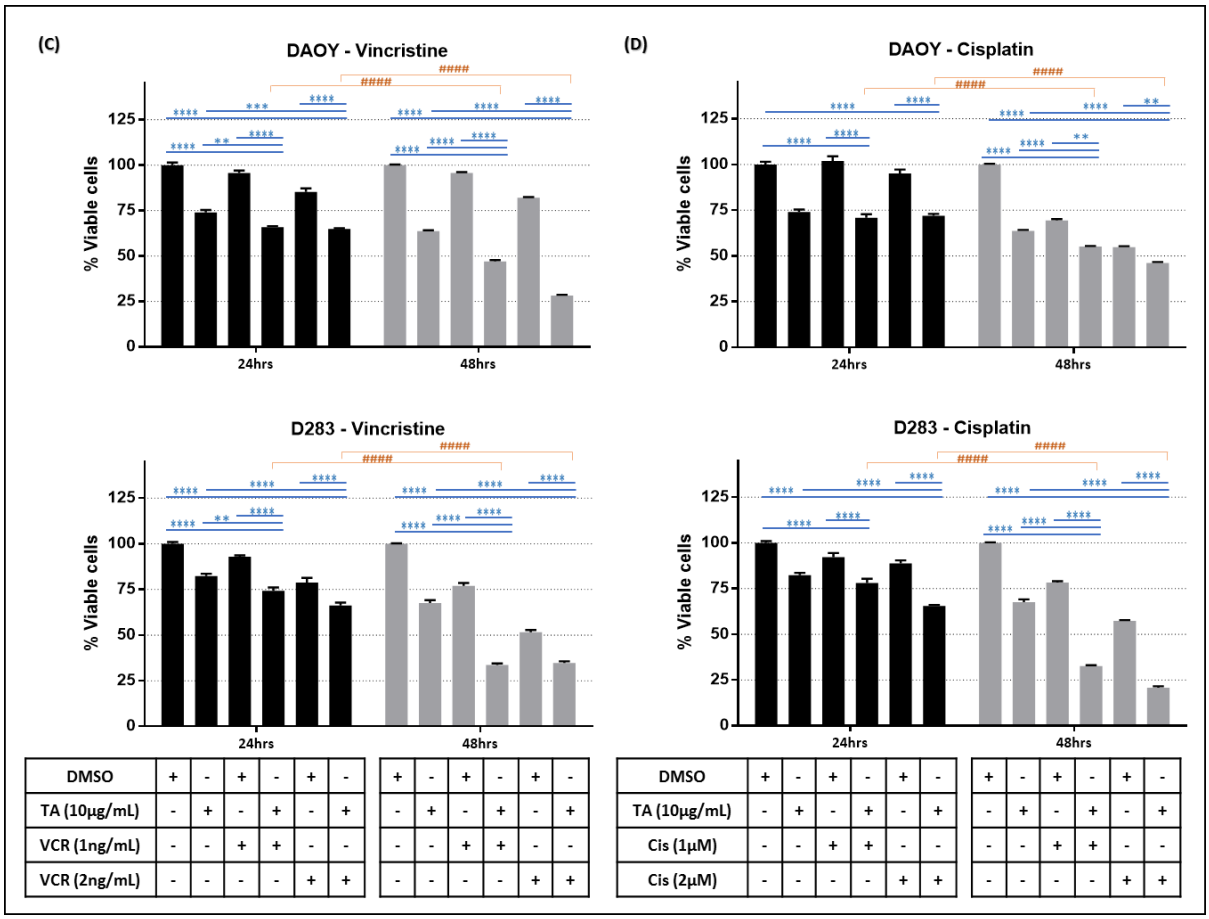
→ Significant difference between timepoints :

# $p<0.05$ ## $p<0.01$ ### $p<0.001$ #### $p<0.0001$

* → Significant difference between treatments:

* $p<0.05$ ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$

(C, D) Combination of TA (10 µg/mL) with VCR (1 or 2 ng/mL) and Cis (1 or 2 µM)



→ Significant difference between timepoints :

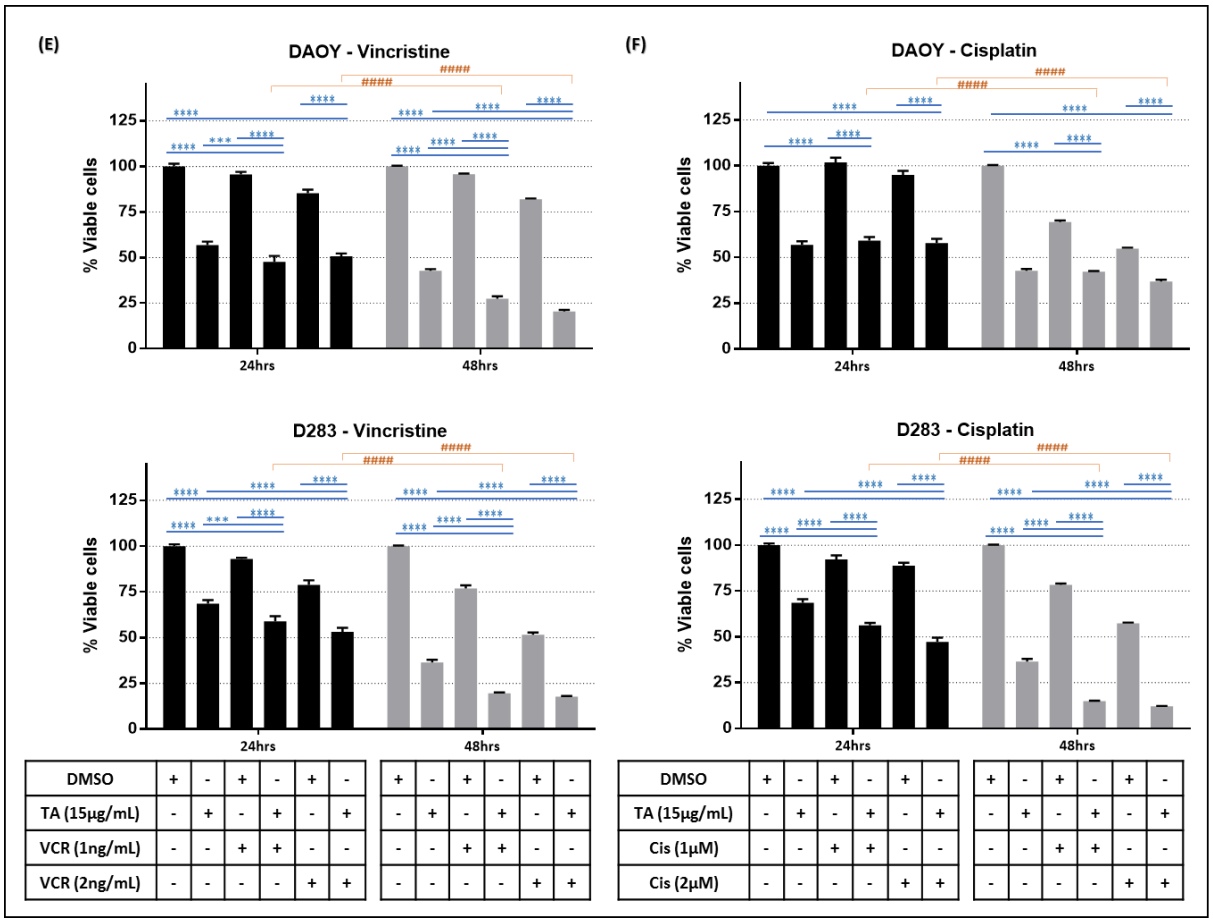
# $p<0.05$ ## $p<0.01$ ### $p<0.001$ #### $p<0.0001$

* → Significant difference between treatments:

* $p<0.05$ ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$

DAOY and D283 were treated with either DMSO (vehicle control) or TA (5 or 10 or 15 µg/mL) or VCR (1 or 2 ng/mL) or Cis (1 or 2 µM) or combination of TA+VCR or TA+Cis. Cell viability was measured at 24- and 48-hours post-treatment using CellTiter-Glo kit (Promega). Percent viable cells over control were calculated and plotted against each treatment doses.

(E, F) Combination of TA (15 µg/mL) with VCR (1 or 2 ng/mL) and Cis (1 or 2 µM)



→ Significant difference between timepoints :

# $p<0.05$ ## $p<0.01$ ### $p<0.001$ #### $p<0.0001$

* → Significant difference between treatments:

* $p<0.05$ ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$

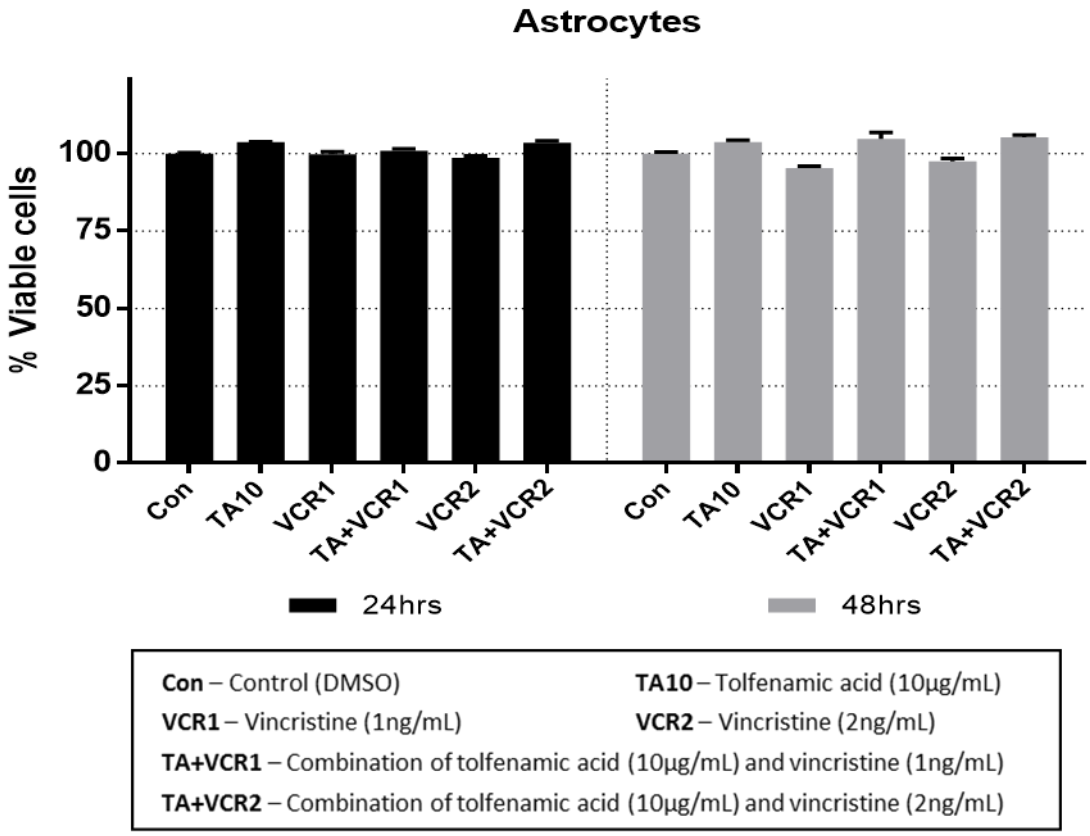
The twelve different graphs shown signifies the combination of VCR **(A, C, E)** and Cis **(B, D, F)** doses with 5, 10 and 15 µg/mL of TA, respectively. Data represents mean ± SEM of three independent observations. The cytotoxic effect of indicated combination treatments with the respective single treatment and control is considered significant as determined via Two-way ANOVA and post-hoc analysis.

Table 3.3: **Combination index value for tolfenamic acid and vincristine co-treatment in medulloblastoma cells.**

	DAOY		D283	
	24 hours	48 hours	24 hours	48 hours
TA10 + VCR1	1.038	0.949	1.007	**0.727**
TA10 + VCR2	1.217	**0.774**	0.936	0.868

The table represents the combination index (CI) values for TA (10 µg/mL) and VCR (1 or 2 ng/mL) co-treatments in MB cell lines at 24- and 48-hour time-point. 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. Calcusyn software was employed for this analysis and determination of CI values. The values indicated in red signifies a moderate synergistic effect observed for the specified drug combination and timepoint.

Figure 3.3: **Effect of tolfenamic acid and vincristine combination on viability of normal primary astrocyte cells.**



Primary normal astrocyte cells (number 2733, 127th day, female donor) obtained from Dr. Ghorpade's lab were treated with either DMSO (Control) or TA (10 µg/mL) or VCR (1 or 2 ng/mL) or TA+VCR. Cell viability was measured at 24- and 48-hours post-treatment using CellTiter-Glo kit (Promega). Percent viable cells over control were calculated and plotted against each treatment doses. Data represents mean ± SEM of three independent observations.

DISCUSSION

VCR and Cis are conventional chemotherapeutic drugs and are clinically used to treat several malignant cancers, including MB [11]. VCR (also known as leurocristine or Oncovin) is a vinka alkaloid that blocks mitosis by binding to the micro-tubulin protein of mitotic spindle apparatus [12]. Cis (*cis*-diamminedichloroplatinum) belongs to the platinum-based family of medications that causes DNA damage. It blocks DNA replication by crosslinking with purine bases and consequently induces apoptosis [13]. Irrespective of their extensive and successful use in treating childhood cancers, the long-term side effects induced by these chemotherapeutic agents is of primary concern as they impede the quality of life of the survivors [14]. The common side effects caused by VCR are peripheral neuropathy including paresthesia, hyponatremia, and hair loss. Cis, however, induces toxicities like nephrotoxicity, ototoxicity and myelosuppression [2,4,15,16]. We postulated that the resulting toxicity of the conventional chemotherapy is dose dependent. To address this issue, we adopted adjuvant therapeutic strategy to enhance the efficacy of these anti-cancer drugs by reducing their toxic doses. Several small molecule NSAIDs are being studied as potential adjuvants in cancer therapeutics [17]. Our group and others have established TA as a potent anti-cancer agent with the potential to increase the effectiveness of standard anti-neoplastic agents [18,19]. In the present study, we tested the anti-proliferative efficacy of VCR and Cis when combined with TA. While we found that TA significantly enhanced the growth inhibitory ability of both VCR and Cis, the combination of TA and VCR showed a synergistic effect consistently in both MB cell lines that were tested.

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 in time and dose dependent manner. Astrocytes are type of glial cells present in abundance in the CNS with diverse physiological properties specific to their location. They are also known to be highly heterogeneous with respect to their shape and functions [20]. The donor cells tested in this study were not affected by the cytotoxic efficacy of the combination doses as demonstrated by the cell viability assay. In addition, as a suggestive goal for future pre-clinical studies, the overall specificity and efficacy of TA+VCR treatment could be further tested on mouse xenograft models for MB.

CONCLUSION

We were able to identify the effective combination dose for DAOY and D283 cell lines from the cell viability based screening assays and selection criterion. This combination synergistically and exclusively enhanced the anti-proliferative activity in MB cells. We found that, TA significantly enhances the cell growth inhibition efficiency of VCR in DAOY and D283 cell lines at drug concentration of less than the IC_{50} value of the individual drug, in a time- and dose-dependent manner. However, we observed such enhanced effect for TA and cisplatin combination treatment only in D283 cell line suggesting its anti-proliferative specificity towards certain molecular sub-type of MB. Additionally, the combination of TA and VCR did not induce any cytotoxic effect in non-cancerous astrocyte cells, serving the purpose of this preliminary study.

Based on the results obtained, we finalized the following combination doses to further investigate the mechanisms inducing the anti-proliferative effect in MB cell lines:

	DAOY	D283
Tolfenamic Acid (TA)	10 ug/mL	10 ug/mL
Vincristine (VCR)	2 ng/mL	1 ng/mL
Combination	TA + VCR	

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CHAPTER – IV

To Evaluate Treatment with Combined Effect of Selected Tolfenamic Acid and Chemotherapeutic Agent Doses on Apoptosis and Cell Cycle Phase Distribution in Medulloblastoma Cell Lines

INTRODUCTION

Our previous studies on several cancer models have demonstrated induction of apoptosis as a contributing factor towards the anti-proliferative activity of TA (tolfenamic acid), with survivin being its potential target [1–5]. Therefore, we assume that an adjuvant treatment of VCR with TA could lead to increase in fraction of apoptotic cell population. This might also cause the up-regulation and down-regulation of specific markers associated with activation and advancement of apoptotic pathways like pro-apoptotic protein, c-PARP (cleaved Poly (ADP-ribose) polymerase) [6]. Other critical markers include caspase 3 and caspase 7, the crucial executioner proteases that are activated at the later stage of apoptosis, either dependent or independent of cytochrome-c release from mitochondria [7,8]. They execute specific catalytic cleavage of several key cellular proteins leading to the formation of apoptotic bodies. In addition, their activity is known to be regulated by survivin, which belongs to the inhibitor of apoptosis (IAP) gene family [9,10].

Moreover, commitment of the cell towards apoptotic cell death can be initiated via disruption of any standard cellular processes, which includes arrest of cells in either of the three cell cycle phases (G_o/G_1, S or G_2/M) [11,12]. Studies published from our laboratory have also illustrated that the TA treatment significantly induces arrest of cell cycle progression at G_0/G_1 phase [2,3]. This effect by TA was suggested to be an early event that

impacted DNA synthesis. In contrast, vincristine (VCR), the microtubule interfering agent, is known to induce cell cycle arrest at G_2/M phase [13]. This chemotherapeutic drug belongs to the class of vinka alkaloids that prevents the polymerization of microtubules during metaphase leading to cell cycle arrest at G_2/M phase [14]. Thus, evaluating the combined effect of these two drugs on cell cycle progression is essential to establish its anti-proliferative effect. Furthermore, survivin is known to be a bifunctional protein that regulates cell cycle progression by influencing microtubule stability [15]. Hence, in view of these facts, determining the effect of combination treatment on survivin expression will be crucial for justifying the purpose of this study.

The present study was designed to analyze the anti-proliferative mechanisms of selected combination doses of TA and VCR in comparison to the individual doses in medulloblastoma (MB) cells. To begin with, we determined the increase in apoptotic cell population by Annexin-V staining induced by TA+VCR combination treatment. Loss of plasma membrane integrity is one of the earliest and characteristic morphological feature of the apoptotic program. Phosphatidylserine (PS), a membrane phospholipid normally found on the cytosolic side, is translocated to the outer segment of the plasma membrane during this process [16]. Annexin-V (36 kDa) protein has high affinity for PS exposed on cellular surface and binds to such phospholipids in Ca^{+2}-dependent manner [17]. Annexin-V is usually conjugated with fluorochromes like Phycoerythrin (PE) which aids in identifying the apoptotic cell population [18]. We have also assessed the activity of effector caspases, along with examining the effect on protein expression of other apoptosis markers and survivin by Western blot analysis. Subsequently, propidium iodide (PI) staining was employed to evaluate the effect of combination doses on cell cycle progression. Progression of cell cycle is majorly controlled by set of cyclin dependent kinases (CDKs), which are activated by their

associated cyclins [19]. Assessment of protein expression of these cell cycle markers further aided in comprehending the effect of combination treatment on cell cycle.

MATERIALS AND METHODS

Antibodies and Reagents:

Primary antibodies against c-PARP, cyclin A and D3 were acquired from Cell Signaling Technologies (Beverly, MA), survivin from R&D Systems (Minneapolis, MN), cyclin B1, CDK4/6 and Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse (secondary) antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and β-actin from Sigma Chemical Co. (St. Louis, MO). Cell lysis buffer was purchased from Invitrogen (Carlsbad, CA) and protease inhibitor from Sigma Chemical Co. BCA (bicinchoninic Acid) protein estimation kit and SuperSignal West Dura Extended Duration Substrate were obtained from Thermo Fisher Scientific (Waltham, MA). Cell apoptosis detection kit, Annexin-V-PE (Phycoerythrin)/7-AAD (7-Amino-Actinimycin), was bought from BD Bioscience (San Jose, CA), and Caspase-Glo 3/7 kit was obtained from Promega (Madison, WI).

Cell Apoptosis detection using Flow Cytometry:

The percentages of apoptotic cell population resulting from the given dose of DMSO (control) or TA or VCR or TA+VCR were measured using Annexin-V-PE/7-AAD apoptosis detection kit (BD Biosciences). The MB cells were plated in 100 mm culture plates and treated with either DMSO, TA (10 µg/mL), VCR (DAOY – 2 ng/mL; D283 – 1 ng/mL) the following day. The treated cells were then collected at 24- and 48-hour timepoints to obtain single cell suspension and briefly washed with PBS (phosphate buffered saline). These cells

were resuspended in 1X binding buffer and further incubated in dark with annexin V-PE antibody and 7-AAD for 20 minutes at room temperature. For staining control, cells from one untreated plate and one treated plate incubated with high dose of TA and VCR (positive control) were harvested and mixed. This mixture of live and dead cells was divided into three parts for three staining controls: unstained cells and cells incubated alone with either annexin V-PE antibody or 7-AAD. After this staining process, the cells were analyzed using Beckman Coulter FC500 flow cytometer, followed by data analysis using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR). The staining controls were used for flow cytometry fluorescence compensation purposes using CXP software V2.2 (Beckman Coulter, Brea, CA). This compensation process was used to normalize the spectral overlap of the two fluorochromes (PE and 7-AAD) used in this assay [20]. The processed data was then represented as percentage of (early or late) apoptotic or non-apoptotic cells in the analyzed cell population. The assay was replicated four times to check the reproducibility of the data and fold increase in apoptotic cell population with respect to the control was calculated for each set. The quadruplet data was shown as mean ± SEM.

Caspase 3/7 Activation Assay:

The effect of the individual and combination treatment on the activity of the effector caspases was assessed using Caspase-Glo 3/7 kit (Promega, Madison, WI) as per the instructions provided by the supplier. The assay set-up, culture conditions and treatment procedures employed were same as described earlier for the cell viability assay in chapter number III. In addition, both the assays were performed in parallel, in same culture plate, to determine the relative caspase activity with respect to the cell death, in time- and dose-dependent manner. The treated cells were incubated with 100µL of assay reagent in dark

for 60 minutes for signal development and luminescence is measured using SYNERGY HT microplate reader (Biotek) at the wavelength of 450nm. The measured luminescence values correspond to the caspase 3 and 7 activity of various treatments. The readings obtained were normalized to vehicle control and was presented as fold change in caspase activity. All the treatments were performed in triplicates and the data was represented as mean ± SEM.

Cellular Protein Extraction and Protein Quantification:

Total cellular protein extracts were prepared using Invitrogen cell lysis buffer (Carlsbad, CA) and quantified to evaluate the effect of single and combination treatments on protein expression. The cells plated in 100mm plates and incubated with DMSO, TA, VCR and TA+VCR at previously determined concentrations for 24- and 48-hours were briefly collected and centrifuged (for 5 minutes at 2,000 rpm at 4 ^{o}C). The cell pellets were then washed once with cold PBS and re-suspended in cold cell lysis buffer supplemented with protease inhibitor (Sigma Chemical Co., St. Louis, MO). This mixture was incubated for 40 minutes on ice with intermittent vortexing every 5-10 minutes. The mixture was then centrifuged at 12,000 rpm for 15 minutes at 4 ^{o}C to obtain the cellular lysate (supernatant) which was stored at -20 ^{o}C for further use. The estimation of total cellular protein concentration in the lysate was determined using BCA protein assay kit (Thermo Fisher Scientific Waltham, MA).

Western Blot Analysis:

Protein lysate obtained from the DMSO or TA or VCR or TA+VCR treated cells were separated via SDS-PAGE and analyzed for protein expression of apoptosis and cell cycle markers through Western blot technique. For SDS-PAGE (sodium dodecyl sulfate – poly-acrylamide gel electrophoresis), 25µg of protein was boiled with loading buffer to be separated on 10% polyacrylamide gel with SDS. The protein bands were then transferred to

a nitrocellulose membrane using iBlot transfer system (Invitrogen, Carlsbad, CA) and blocked with blocking buffer, i.e. 5% non-fat dry milk *(w/v*) in TBST (tris-buffered saline with 0.1% Tween 20; 10mMol/L Tris pH 7.6, 10mMol/L sodium chloride) at room temperature for 1-hour, to avoid non-specific binding of antibodies. Further, the blocked blots were probed overnight at 4 ^{o}C with specific primary antibodies of apoptotic markers (c-PARP and survivin] and cell cycle markers [Cyclin A, B1, D3, CDK4/6] (Santacruz Biotechnology, Santacruz, CA or Cell Signaling Technology, Beverly, MA or R&D Systems, Minneapolis, MN). The expression of β-actin (Sigma Chemical Co., St. Louis, MO) was used as a loading control. These membranes treated with primary antibodies were washed with TBST and incubated for 2-hours with their respective HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed again with TBST before getting developed by SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific Waltham, MA). The images of respective protein bands were acquired using BioSpectrum Imaging System (UVP, Upland, CA).

Flow Cytometry Analysis of Cell Cycle:

To evaluate the cell cycle phase distribution of treated cells via the flow-cytometric analysis, the MB cells were stained with propidium iodide, a DNA intercalating agent. The intensity of the PI stain was equivalent to the DNA content in each three phases of cell cycle. DAOY and D283 cells were plated in a 6-well culture plate at 75,000 and 113,000 cells per well per 2mL, respectively, followed by treatment with the specified individual and combination doses of TA and VCR. The cells were harvested at 12-, 24- and 48-hours post-treatment to obtain single cell suspension and later washed by PBS. These cells were then fixed in cold 70% ethanol overnight at -20^{o}C. The fixed cells were centrifuged at 400*g* for 5 minutes and

washed with PBS. Further, these cells were resuspended in PI buffer (0.20µg/mL PI, 20µg/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 Beckman Coulter FC500 flow cytometer. The data obtained was analyzed using FlowJo software V8.0 (Tree Star, Inc., Ashland, OR) and represented as percent cells in three different phases of cell cycle, G_0/G_1, S and G_2/M. The cell cycle assay was replicated for four times and fold increase in the percentage of arrested cells in each phase with respect to the control was calculated exclusively for all the sets. The quadruplet data was shown as mean ± SEM of the fold change values.

Statistical Analysis:

The statistical significance of caspases activity data, fold change in apoptotic cell population and for number of cells arrested in cell cycle phases were also determined by two-way ANOVA with respective post-hoc analysis (Tukey's and Sidak's) as mentioned in 'statistical analysis' section of chapter III. *P* value < 0.05 was considered statistically significant.

RESULTS

Combination of Tolfenamic Acid and Vincristine Induces Apoptosis in DAOY and D283 Cells:

We gauged the increase in apoptotic cell population via flow cytometric analysis to determine the mechanisms involved in anti-proliferative effect induced by the combination treatment of TA10 (10 µg/mL) with VCR2 (2 ng/mL) or VCR1 (1 ng/mL) in DAOY and D283 cells, respectively. For comparison purposes, MB cells were also treated with control (DMSO) and their respective individual treatments. The apoptotic cell population were measured using Annexin-V-PE/7-AAD kit (BD Biosciences) at 24- and 48-hour post-treatment

(Figure 4.1). Annexin-V and 7-AAD positive cells represented late apoptotic cells (LA), whereas, only annexin-V positive cells symbolized early apoptotic cells (EA). Cells negative to both stains correspond to live cells. Interestingly, we observed that the co-treatment caused significant (p<0.05) dose-dependent increase in annexin-V labeled cells. When compared with non-treated cells, the early and late apoptotic cell population increased by 2.7-fold and 2-fold at 48-hours post-treatment in DAOY and D283 cell lines, respectively. Further, the data illustrates that the percentage of apoptotic and dead cell population were more in combination treatment compared to the respective single treatments.

Further, we evaluated the combination effect on the activity of key effector caspases, also known as the hallmark of apoptosis induction. The changes observed in caspase 3/7 activity correlates with the cell viability data (Figure 4.2 A & B). We found that, TA and VCR co-treatment significantly increased the caspase 3/7 activity compared to the individual treatment in time-dependent manner, as assessed by CaspaseGlo-3/7 assay kit (Promega). In DAOY cells, about 15-fold and 19-fold increase in caspase activity was observed at 24- and 48-hours, respectively. On the other hand, the upsurge of caspase 3/7 activity noted in D283 cells was 3.6-fold (24hr) and 9.7-fold (48hr) compared to control.

Moreover, these observations are consistent with the increase in cleaved-PARP expression, which is the downstream target of activated signaling cascade of apoptotic pathway (Figure 4.2C). As determined by Western blot analysis, increase in c-PARP level was observed in both DAOY and D283 cells treated with the combination doses at 24- and 48-hours post-treatment. The individual treatments did not induce a major increase of this cleaved protein in D283 cells and after 24-hour treatment in DAOY cells. However, VCR2 treatment caused a slight raise in c-PARP protein level at 48-hours. On the whole, these results suggest that

induction of apoptosis is one of the crucial mechanisms leading to the enhanced anti-proliferative activity of the proposed combination treatment.

Tolfenamic Acid and Vincristine Combination Treatment Decreases Survivin Expression in DAOY and D283 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 demonstrated survivin as a potential therapeutic target of TA. Hence, we evaluated the expression of survivin by Western blot analysis, in an attempt to understand the anti-proliferative mechanism of the combination treatment. As shown in Figure 4.2C, the co-treatment of TA10 (10 µg/mL) and VCR1/2 (1 or 2 ng/mL) down-regulated the expression of survivin in both DAOY and D283 cells. This decrease in survivin expression at 48-hours correlates with the growth inhibitory response of TA+VCR treatment. However, the current data is insufficient in suggesting a complete mechanism involved in regulation of survivin expression.

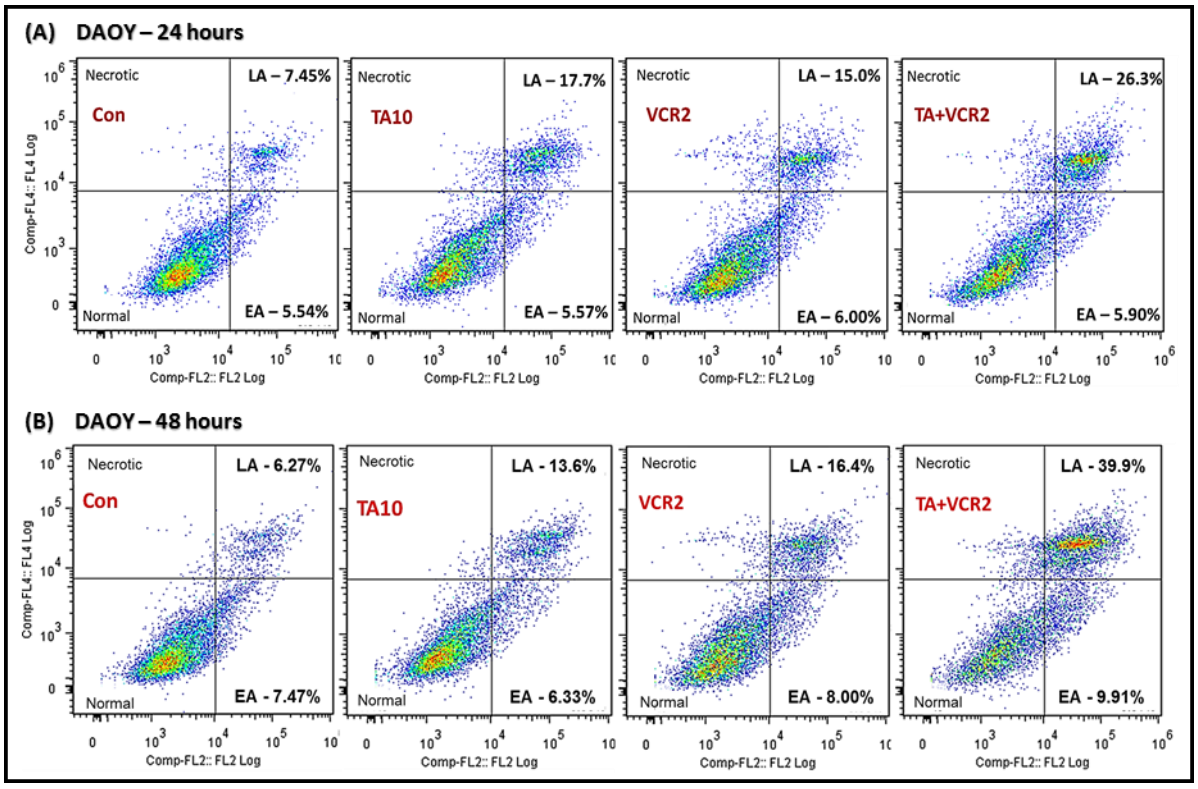
Combination of Tolfenamic Acid and Vincristine Causes Cell Cycle Arrest in DAOY and D283 Cells:

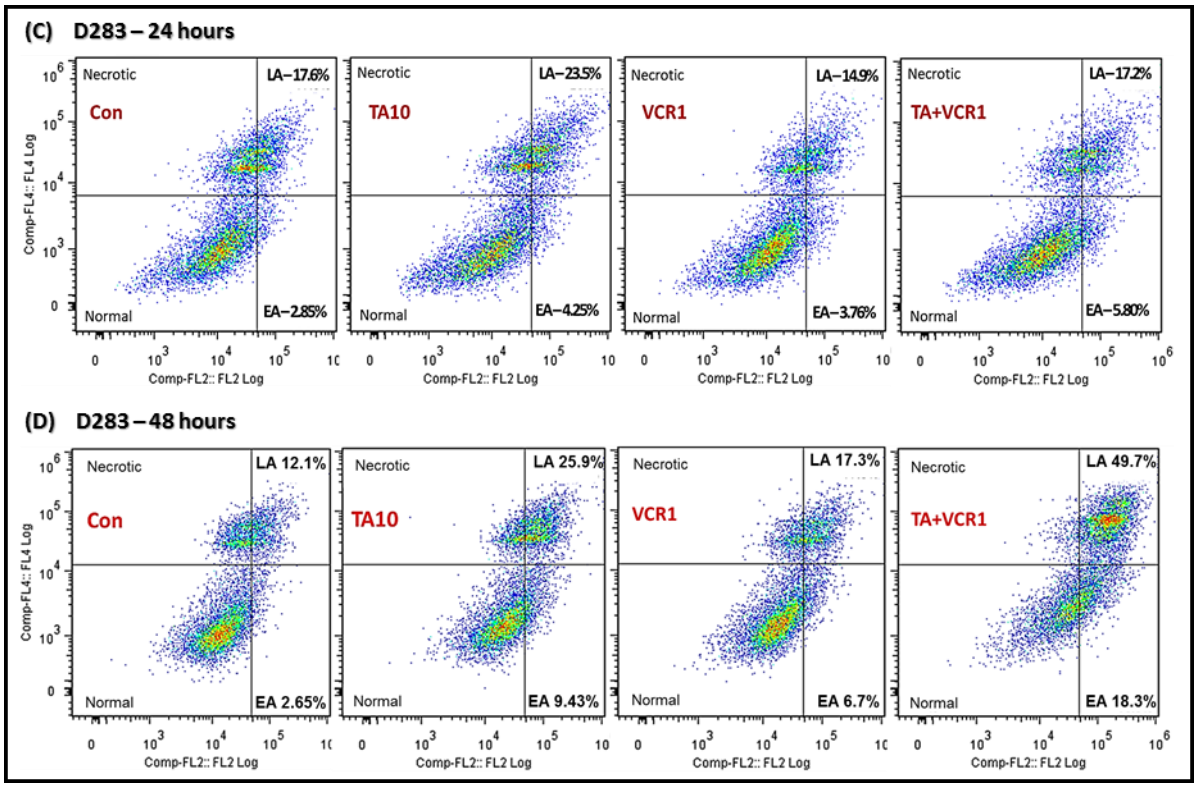
Apart from induction of apoptosis, cell cycle arrest could also be a potential mechanism in decreasing the cell viability of MB cells co-treated with TA+VCR. Therefore, we analyzed the proportions of cells at various phases of the cell cycle by measuring the cellular DNA contents (stained with PI) using flow cytometer. The effect of the individual and combination treatment was evaluated at 12-, 24- and 48- hour post treatment (Figure 4.3). As presented in Table 4.1 and 4.2, TA+VCR treatment at 12-hour caused 1.4-fold increase in

G_2/M phase arrest for both DAOY and D283 cell lines, along with minor increase in S phase (by 1.2-fold in DAOY cells and 1.3-fold in D283 cells). We further observed about 1.8-fold increase in the number of cells arrested in G_2/M phase at 24- and 48-hour time-points. However, a minimal increase in G_0/G_1 and G_2/M phase arrest was observed for individual treatment of TA and VCR, respectively. This observation indicates that cell cycle arrest is an early event that induces an anti-proliferative response in co-treated MB cells.

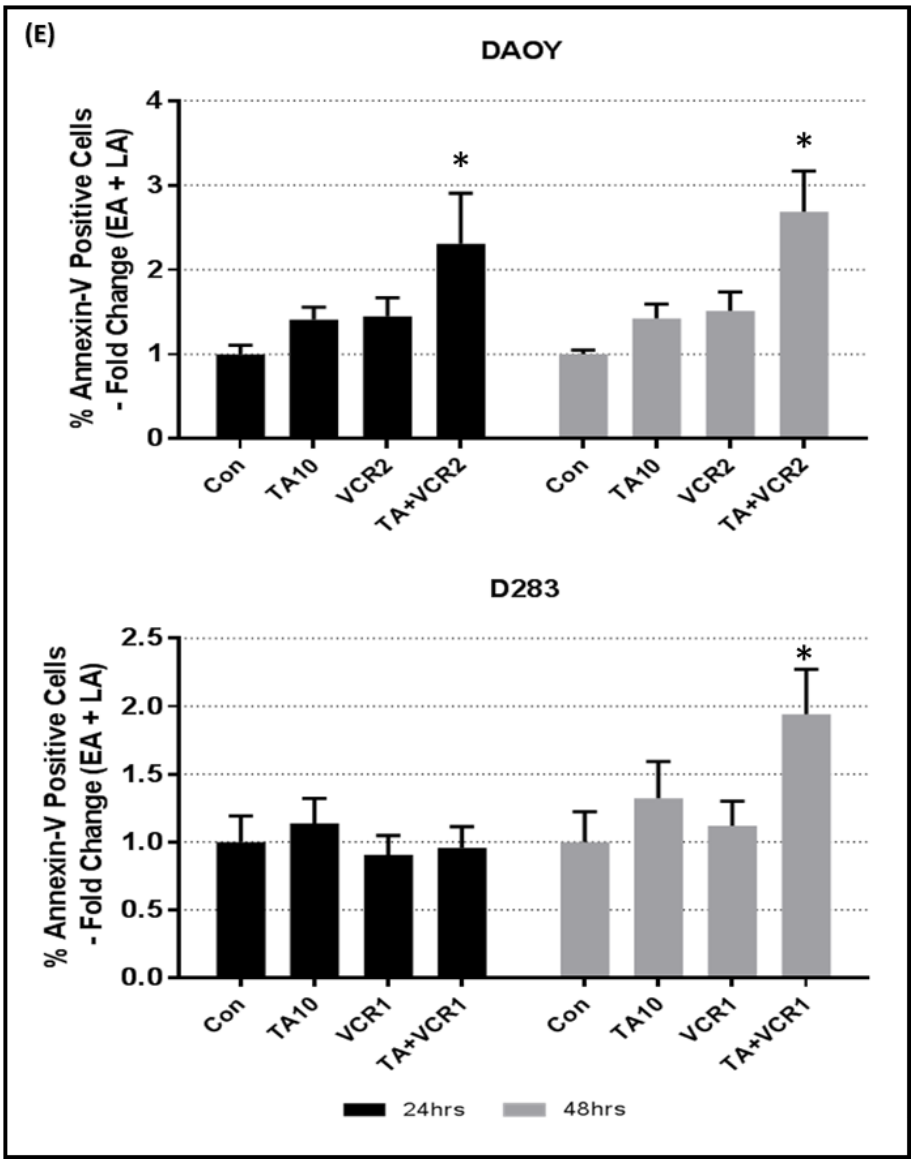
It is known that the progression of the cell cycle through its specific checkpoints is primarily regulated by the cyclins-CDK complexes. Any alteration in the threshold of these protein kinases leads to the cell cycle arrest [21]. To determine the cause of G_2/M arrest due to combination treatment we evaluated the 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 the cell lines at 48-hour, when compared to the control and individual treatments (Figure 4.4). 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.

Figure 4.1: **Combination of tolfenamic acid and vincristine causes increase in apoptotic cell population of medulloblastoma cell lines.**





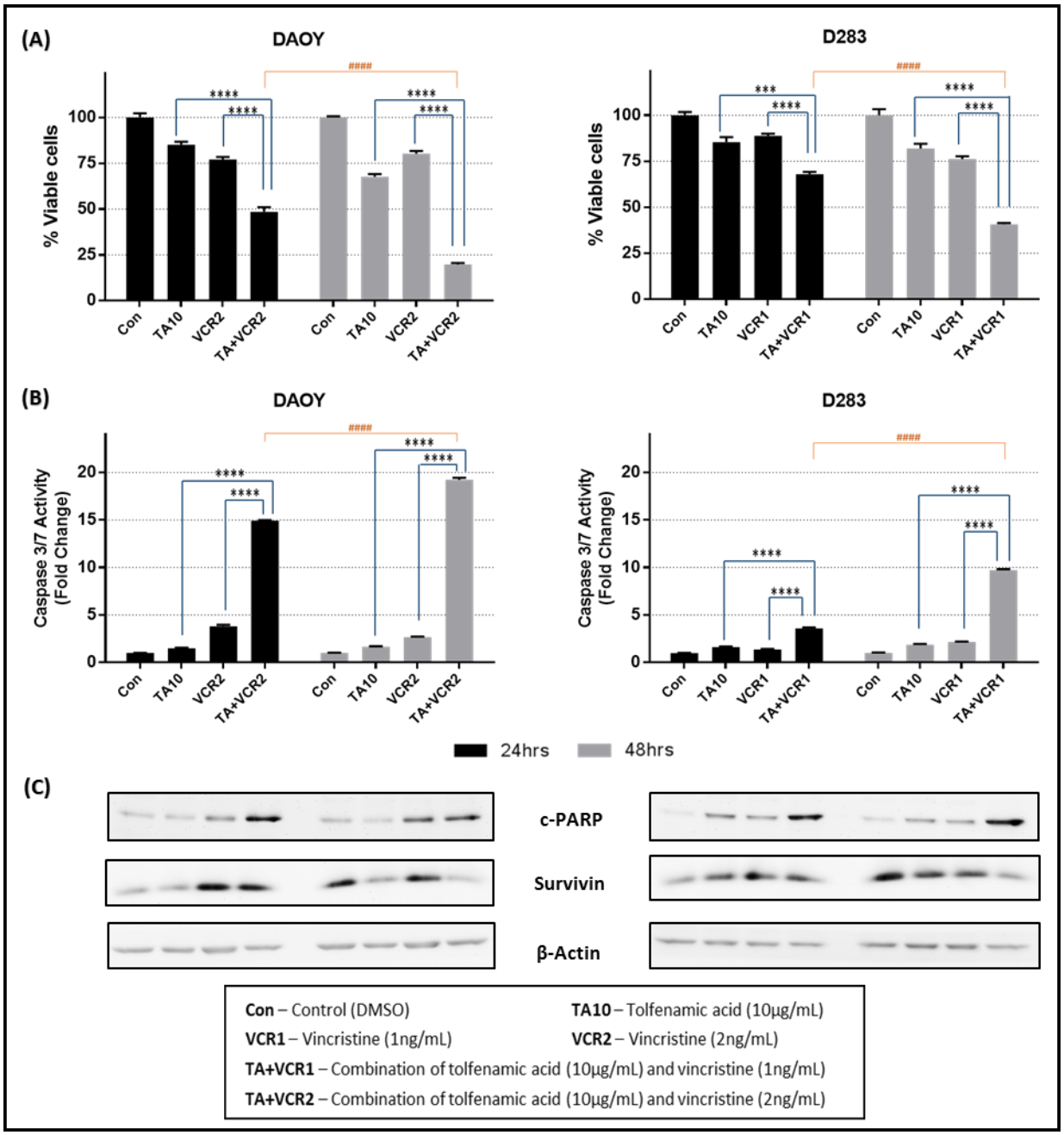
The number of cells undergoing apoptosis was determined by flow cytometry analysis using Annexin-V-PE/7-AAD kit (BD Biosciences). MB cells were treated with DMSO (Control) or TA (10 µg/mL) or VCR (DAOY: 2 ng/mL; D283: 1 ng/mL) or both. **(A-D)** Apoptotic [PE-Annexin-V positive (FL2 log – X-axis)] and dead [7-AAD positive (FL4 log – Y-axis)] cell populations were assessed at 24- and 48-hour post-treatment. the X- and Y-axis represents the intensity of the respective fluorescent flow-cytometric dyes. *(EA – Early Apoptotic cells; LA – Late Apoptotic cells).* Data shown are the representative result from four independent determinations.



Con – Control (DMSO)	**TA10** – Tolfenamic acid (10µg/mL)
VCR1 – Vincristine (1ng/mL)	**VCR2** – Vincristine (2ng/mL)
TA+VCR1 – Combination of tolfenamic acid (10µg/mL) and vincristine (1ng/mL)	
TA+VCR2 – Combination of tolfenamic acid (10µg/mL) and vincristine (2ng/mL)	

(E) The bar graphs signify the fold change in percentage of apoptotic cell population (EA cells + LA cells) with respect to the treatments. Data represent mean ± SEM of four independent observations. Overall, the results signify that the combination doses increase the percentage of apoptotic cell population compared to control and respective individual treatments. * represent a significant (p<0.05) increase in apoptotic cell population compared to the control, determined by two-way ANOVA statistical analysis.

Figure 4.2: **Tolfenamic acid and vincristine combination increases cell death and caspase 3/7 activity in medulloblastoma cell lines, accompanied by modulation of c-PARP and survivin expression.**



MB cells were treated with DMSO (Control) or TA (10µg/mL) or VCR (DAOY: 2ng/mL; D283: 1ng/mL) or both. **(A)** Cell viability, **(B)** caspase 3/7 activity were determined at 24- and 48-hour post-treatment using CellTiter-Glo and Caspase-Glo-3/7 kits (Promega), 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 represent mean ± SEM of three independent observations. All groups are significantly different from corresponding controls (p<0.01). The effect of combination treatment significantly increases at 48-hour compared to the individual treatments as indicated (p<0.0001). **(C)** Protein extracts were prepared from the treated cells as described above and separated via SDS-PAGE. Protein expression of c-PARP and survivin was evaluated by Western blot analysis, wherein, β-actin was used as a loading control. The data were obtained from at least three different determinations and representative gels are shown in the figure.

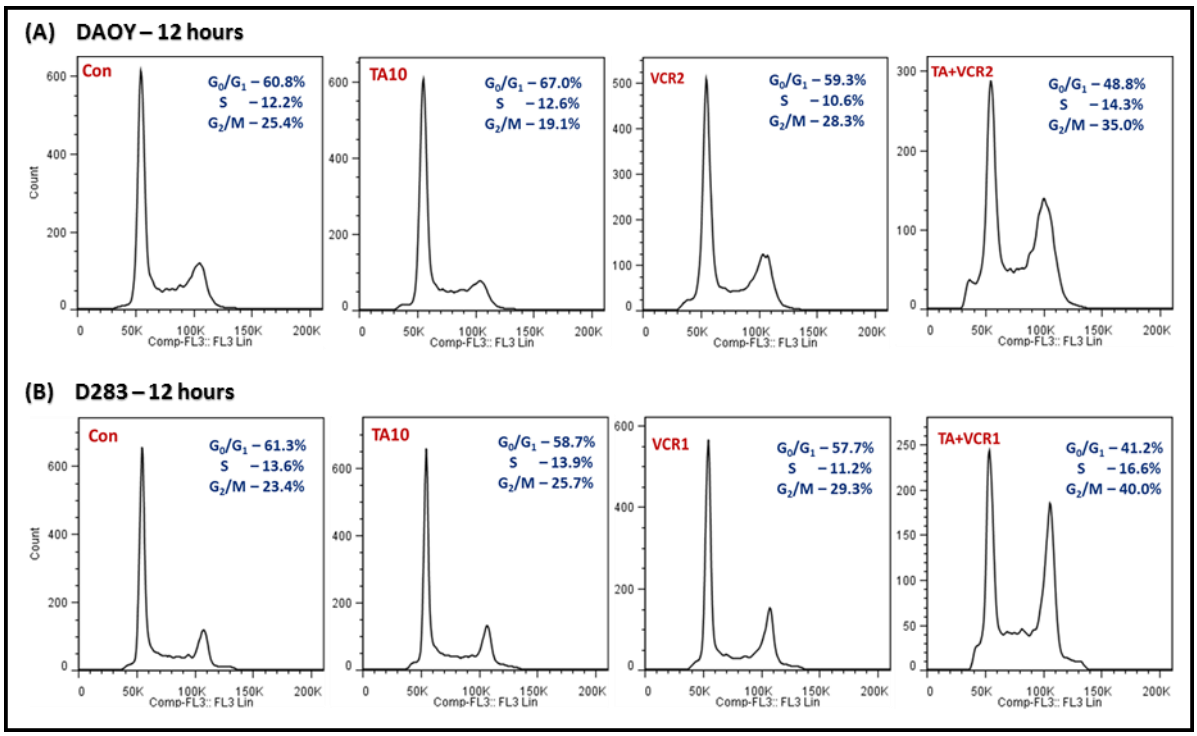
Table 4.1: **Effect of tolfenamic acid and vincristine combination on cell cycle phase distribution of medulloblastoma cell lines.**

(A)		DAOY - Cell Cycle Fold Change in % Cell Count					
		12 hours		24 hours		48 hours	
		Mean	SEM	Mean	SEM	Mean	SEM
G_0/G_1	Con	1.0	0.0	1.0	0.0	1.0	0.0
	TA10	1.1	0.0	1.1	0.0	1.0	0.0
	VCR2	1.0	0.0	1.0	0.0	0.9	0.0
	TA+VCR2	0.8	0.0	0.9	0.1	0.8	0.1
S	Con	1.0	0.2	1.0	0.1	1.0	0.1
	TA10	1.0	0.1	1.1	0.1	1.1	0.2
	VCR2	0.9	0.1	1.1	0.1	1.2	0.1
	TA+VCR2	1.2	0.2	1.1	0.2	1.2	0.2
G_2/M	Con	1.0	0.1	1.0	0.1	1.0	0.0
	TA10	0.9	0.1	0.9	0.1	1.1	0.0
	VCR2	1.1	0.0	1.1	0.1	1.2	0.0
	TA+VCR2	1.4	0.1	1.2	0.2	1.9	0.4

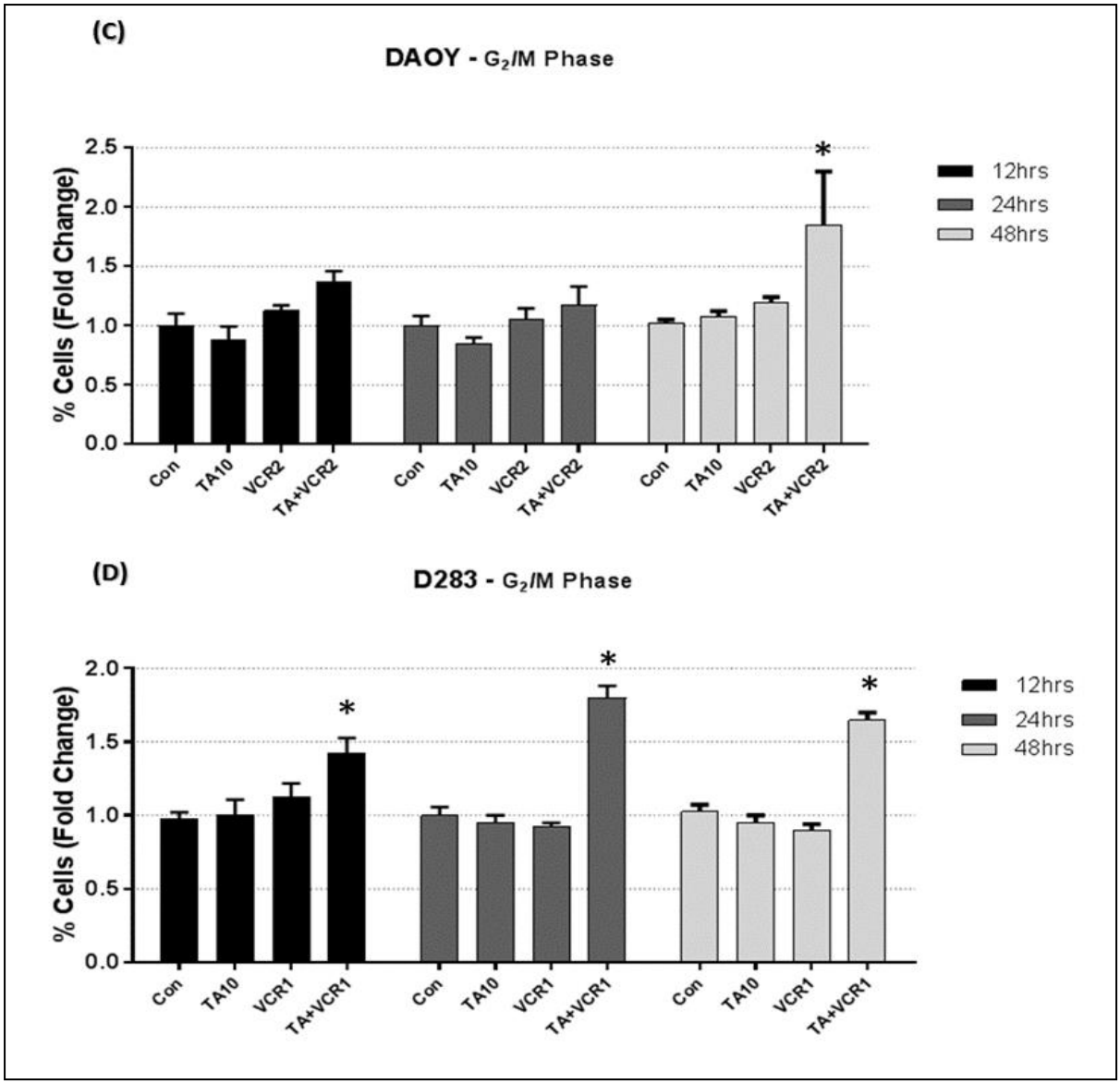
(B)		D283 - Cell Cycle Fold Change in % Cell Count					
		12 hours		24 hours		48 hours	
		Mean	SEM	Mean	SEM	Mean	SEM
G_0/G_1	Con	1.0	0.0	1.0	0.0	1.0	0.0
	TA10	1.0	0.1	1.0	0.0	1.0	0.0
	VCR1	1.0	0.1	1.1	0.0	1.1	0.0
	TA+VCR1	0.8	0.0	0.6	0.0	0.7	0.0
S	Con	1.0	0.0	1.0	0.0	1.0	0.1
	TA10	1.1	0.1	1.1	0.0	1.1	0.0
	VCR1	0.9	0.1	0.8	0.0	0.8	0.1
	TA+VCR1	1.3	0.1	1.0	0.1	1.3	0.0
G_2/M	Con	1.0	0.0	1.0	0.1	1.0	0.0
	TA10	1.0	0.1	1.0	0.1	1.0	0.1
	VCR1	1.1	0.1	0.9	0.0	0.9	0.0
	TA+VCR1	1.4	0.1	1.8	0.1	1.7	0.0

MB cells, **(A)** DAOY and **(B)** D283 cells were treated with DMSO (Control), TA (10 µg/mL) or (DAOY: 2 ng/mL; D283: 1 ng/mL) or both for 12-, 24- and 48-hour. Cell cycle progression was analyzed by flow cytometry after cell fixing and propidium iodide staining. The values represent the fold change in percentage of cells arrested in G_0/G_1, S and G_2/M phase for each treatment. Data represent mean ± SEM of four independent observations.

Figure 4.3: **Combination of tolfenamic acid and vincristine causes cell cycle arrest in medulloblastoma cells.**



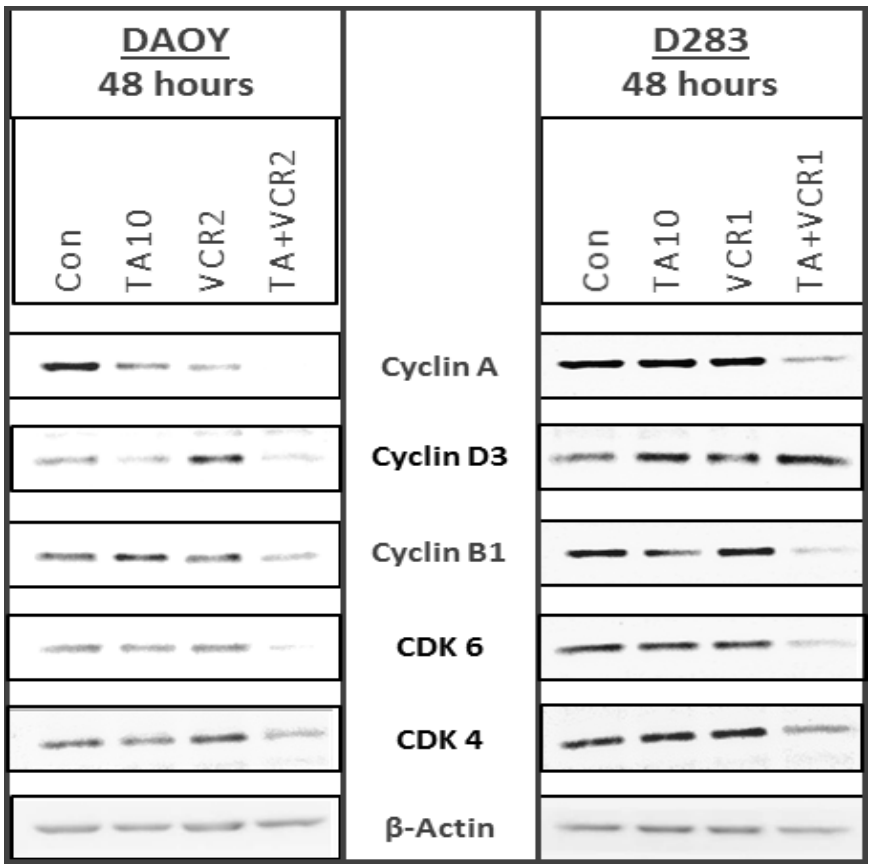
MB cells were treated with DMSO (Control) or TA (10 µg/mL) or VCR (DAOY: 2 ng/mL; D283: 1 ng/mL) or both for 12-, 24- and 48-hours. Cell cycle progression was analyzed by flow cytometry after cell fixing and propidium iodide staining. **(A & B)** Data shown are the representative result from four independent determinations. The X-axis and Y-axis represents intensity of PI stain (representing DNA content) and cell count, respectively.



Con – Control (DMSO)	**TA10** – Tolfenamic acid (10µg/mL)
VCR1 – Vincristine (1ng/mL)	**VCR2** – Vincristine (2ng/mL)
TA+VCR1 – Combination of tolfenamic acid (10µg/mL) and vincristine (1ng/mL)	
TA+VCR2 – Combination of tolfenamic acid (10µg/mL) and vincristine (2ng/mL)	

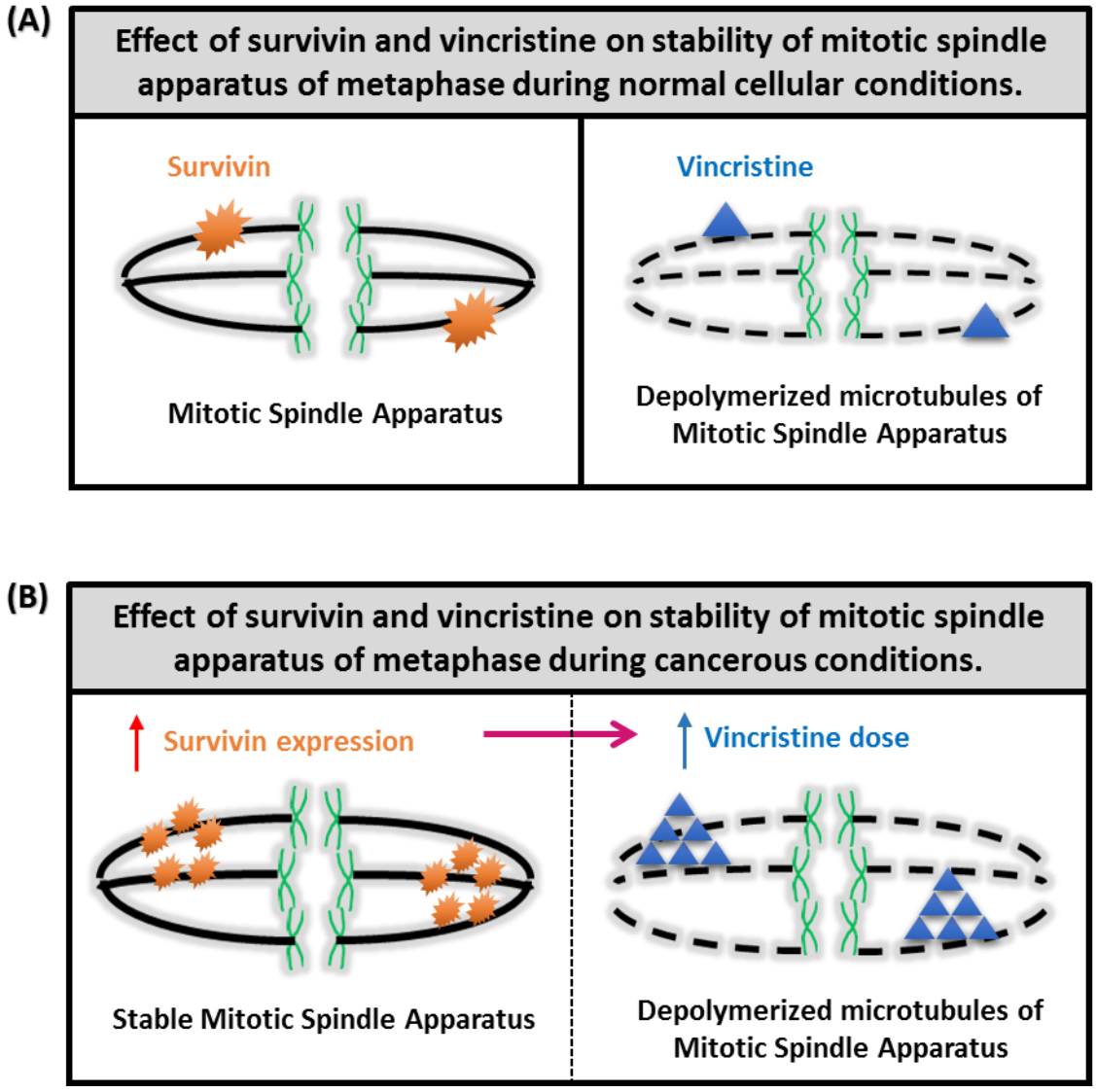
(C & D) The bar graphs signify the fold change in percentage of cells arrested in G_2/M phase for each treatment. Data represent mean ± SEM of four independent observations. The indicated (*) increase in cell count induced by combination treatment are statistically significant (p<0.05) compared to control and individual treatment as determined by two-way ANOVA statistical analysis.

Figure 4.4: **Tolfenamic acid and vincristine combination modulates expression of cell cycle markers in medulloblastoma cell lines.**



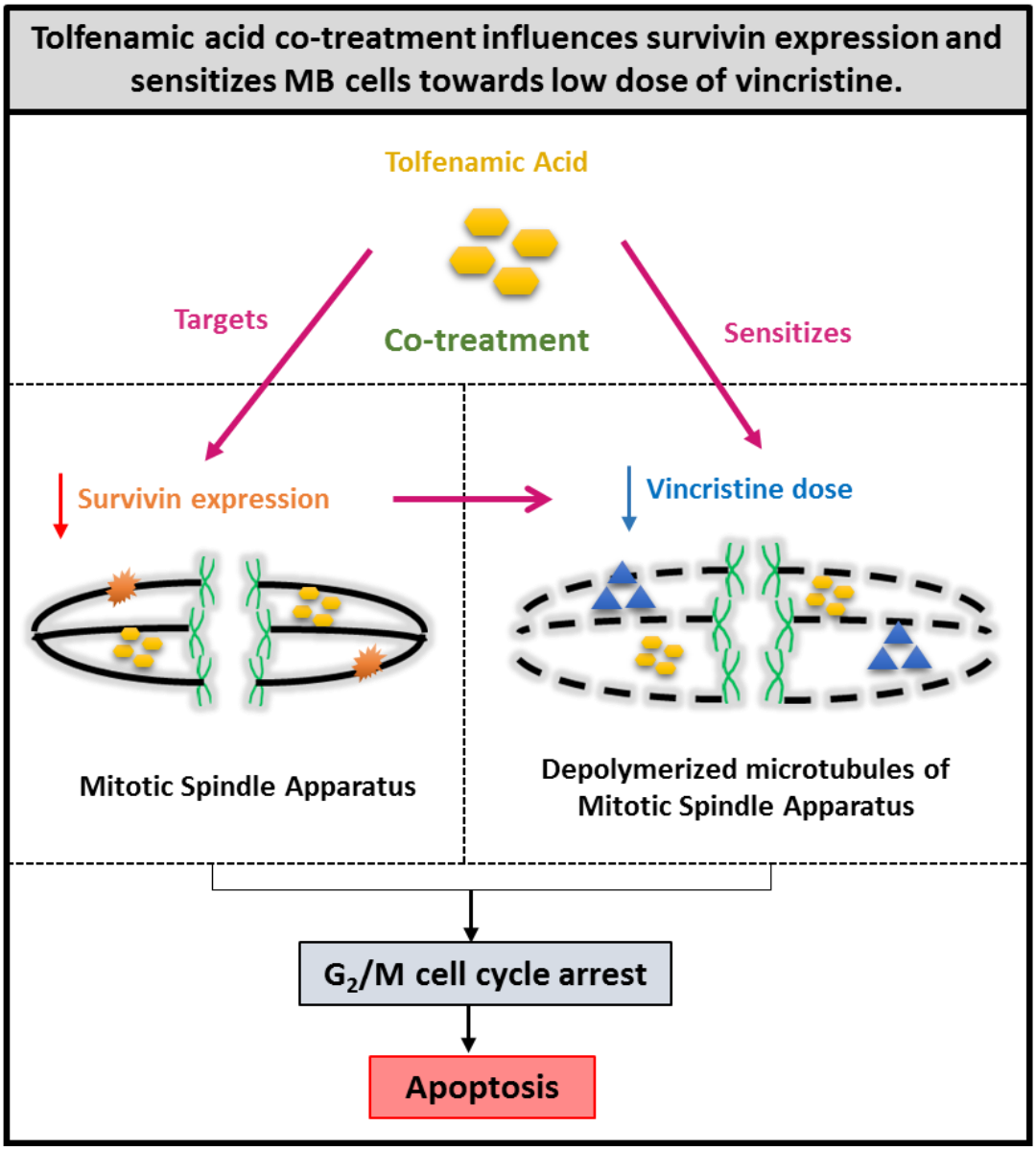
MB cells, DAOY and D283 cells were treated with DMSO (Control), TA (10 µg/mL) or (DAOY: 2 ng/mL; D283: 1 ng/mL) or both for 48-hour to obtain the protein extract. Cell lysates were used for protein separation and evaluation by SDS-PAGE and Western blot analysis, respectively. The expression of cyclin B1, D3, A and CDK 4/6 was evaluated, wherein, β-actin was used as a loading control. The data were obtained from at least three different determinations and representative gels are shown in the figure.

Figure 4.5: ***Proposed Theory*** **– Possible mechanism of action of tolfenamic acid and vincristine co-treatment for inducing cell cycle arrest and apoptosis in medulloblastoma cell lines.**



The figure shows the effect of survivin and VCR on assembly of mitotic spindle apparatus during metaphase in normal and cancer cells. **(A)** During normal cellular conditions, the regulated expression of survivin associates and stabilizes the microtubule filaments of the mitotic spindle apparatus. The chemotherapeutic efficacy of VCR is determined by its microtubule depolymerizing capability during metaphase of mitotic cell division.

(C)



(B) Increased survivin expression is noted for several cancer types including MB, which aids in cell cycle progression of cancer cells and thus inhibiting apoptosis. This may counteract the therapeutic outcome of VCR resulting in use of high VCR dose to cause the depolymerization of microtubules. **(C)** This section illustrates the influence of TA on activities of survivin and VCR. TA co-treatment leads to downregulation of survivin expression and hence sensitizes the MB cells towards VCR resulting in disruption of mitotic spindle apparatus at low dose of VCR. Thus, the combined effect of TA and VCR at low dose leads to cell cycle arrest of MB cells at G_2/M phase and cell death via apoptosis.

DISCUSSION

Evading apoptosis is one of the hallmarks of cancer and thus is the favored target of therapeutic strategies [22]. In the present study, we tried to establish the link between the anti-proliferative activity of combination drug treatment with apoptosis initiation. Under normal physiological conditions, the cellular process of apoptosis is activated via either of the two commonly known pathways, intrinsic pathway and/or extrinsic pathway [7,8]. The intrinsic pathway is primarily initiated due to internal cellular stress like irreparable DNA damage or severe oxidative stress. This eventually results in release of cytochrome-c from the mitochondria due to the increased permeability of mitochondrial membrane. The extrinsic pathway, also known as death receptor pathway, is stimulated via external death signal leading to the formation of death-inducing signaling complex (DISC). Both these pathways further initiate a cascade of proteolytic events, wherein activation of up-stream procaspases and downstream procaspases plays a central role in amplification of apoptotic signals [23]. The release of cytochrome-c and DISC formation ultimately converges towards stimulation of executioner caspases (3/7) via initiator caspases 9 and 8, respectively. Caspase 3 and 7 causes DNA fragmentation, cleavage of cytoskeletal elements and cleavage of several vital cellular proteins including 113 kDa PARP into fragments of 24- and 89-kDa [6]. The selective cleavage of this chromatin-associated enzyme between Asp214 and Gly215 is considered as a universal phenomenon initiating at the early phase of apoptotic programmed cell death mechanism. Our results for combination treatment have demonstrated that the up-regulation of the pro-apoptotic marker, c-PARP, correlates with the increased activity of caspase 3/7 in a time-dependent manner.

In addition, changes to the biochemical features of the outer plasma membrane contribute towards non-inflammatory recognition of the apoptotic cells by the phagocytic cells [24]. This phenomenon is characterized by the expression of the PS on the outer leaflet of the plasma membrane during the early apoptosis phase and is known to be one of the hallmarks of apoptosis [25]. Due to the high binding affinity of annexin-V for PS, identification of apoptotic cell population has become feasible via biochemical assays [17,18]. 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 caused significant increase in annexin-V positive cells. Although, the mechanism of PS translocation from inner to outer segment of the plasma membrane is not yet well understood, some studies have suggested the role of two events, i.e., the loss of amino-phospholipid translocase and activation of scramblases [16]. In normal cellular conditions, the amino-phospholipid translocase (flippase) along with P-glycoprotein (floppase) aids in maintaining the phospholipid asymmetry of inner and outer leaflet of plasma membrane via flip-flop process in ATP-dependent fashion [26]. Scramblases, on the other hand, causes the dissipation of this asymmetric distribution of plasma membrane phospholipids [27]. This ATP-independent scrambling process of plasma membrane exposes PS to the external surface of the cell during early phase of apoptosis. Certain studies have reported that the caspase 3/7 cleavage activates the specific apoptosis restricted scramblases [16]. Based on these evidences we can suggest that the increased caspase 3/7 activity observed for our combination treatment may contribute in activation of these apoptosis associated scramblases. This ultimately results in increased expression of PS on the external surface of the MB cells which are detected as annexin-V positive cells.

Survivin belongs to the IAP (inhibitor of apoptosis) class of gene family and its overexpression is associated with tumor progression, invasiveness and therapeutic resistance leading to poor prognostic outcome in several cancers, including MB [28,29]. Survivin is identified as a potential therapeutic target due to its dual role as inhibitor of apoptosis and regulator of mitosis progression [30,31]. During normal cellular conditions, the regulated expression of survivin is noted during the G_2/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 (Figure 4.5A) [32,33]. This mechanism is also responsible for inducing resistance in cancer cells by counteracting the therapeutic outcome of microtubule depolymerizing drugs like vinka alkaloids (Figure 4.5B) [34]. 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, which also correlates with our data demonstrating an increase in caspase 3/7 activity with downregulation of survivin expression induced by combination treatment [9,10,33]. In addition, the resulting destabilization of these alpha- and beta-tubulin polymers arrests the cells at the M phase, which is regulated by the spindle arrest checkpoint [35]. The study by Brun *et.al.* (2015), also reported that genetic deletion of survivin expression in tumor cell models causes cell cycle arrest at G_2/M phase [36]. The observations of this study can be correlated to the above stated facts, wherein the TA+VCR co-treatment substantially induced G_2/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. Our prior studies have demonstrated the inhibition of survivin by TA in 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 (Figure 4.5C).

Cell cycle regulation is a complex process controlled by the extrinsic (mitogenic signals) and intrinsic (concomitant phosphorylation of cell cycle proteins by protein kinases) stimuli. It is known that 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 [21,37]. Cumulative expression of cyclins and their associated CDK's regulate the progression of cell cycle [12]. Therefore, downregulation of these cyclins or CDK's would determine the block of cell cycle at a certain phase [38]. This study demonstrated that TA+VCR combination noticeably decreased the expression of CDK4 and CDK6, which are homologous serine or threonine specific kinases. When complexed with cyclin D in a tissue-specific manner, they permit the progression of the cell into the DNA synthesis (S) phase primarily by phosphorylating RB (retinoblastoma tumor suppressor) protein that releases the active form of E2F transcription factor [19]. Inhibitors of these CDKs are extensively studied for treating various cancers types by causing arrest at the G_0/G_1 phase [39,40]. Whiteway *et al*. in 2013 have also reported the overexpression of CDK6 in MB as a potential therapeutic target to suppress the cell proliferation [41]. Even though our current CDK4/6 and cyclin D protein expression data do not correlate with the increased G_2/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 G_2/M phase caused by 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 G_2 phase promotes mitosis when coupled with CDK1 [42,43].

While the protein expression patterns of these cyclins and CDKs support the enhanced efficacy of the combination therapy, it is difficult to predict if they are the actual mechanism instigating the synergistic outcome of the combination doses. Alternatively, these expression patterns could possibly be the after-effects of the microtubule destabilization caused by VCR and TA inhibition of survivin. Hence, further investigation elucidating the cell cycle and apoptotic pathways affected by the combination doses is obligatory.

CONCLUSION

Overall, our study suggests that the combination of TA with VCR induced the cell growth inhibition in MB cells, accompanied by apoptosis and cell cycle arrest. To support the association of enhanced anti-proliferative response with apoptosis, we demonstrated the increased activity of apoptotic markers like caspase 3/7 and c-PARP protein expression. In addition, the elevation of apoptotic markers was analogous to the amplified population of the apoptotic cells (annexin-V positive cells) determined by the flipping of PS to the external surface of the plasma membrane initiated during the early phase of the apoptosis pathway. Decrease in IAP expression, i.e. survivin, also contributes towards the overall induction of apoptosis by combination treatment. We further conclude that survivin down-regulation is conceivably the prime reason to sensitize the MB cells towards VCR treatment that results in reduction of its therapeutic dose. Destabilization of the microtubule assembly during mitosis is suggested as the plausible mechanism of action, also resulting in the G_2/M arrest of MB cells. Down-regulation of cell cycle markers like cyclin A, B1 and CDK6/7 was also shown to contribute towards the anti-proliferative efficacy of TA+VCR co-treatment. However, these current findings established for the combination treatment warrants for further investigation to comprehend its mechanism of action.

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CHAPTER – V

Summary and Future Directions

SUMMARY

Medulloblastoma (MB) is among the most prevalent pediatric cancer of the brain. MB originates in the cerebellum and is classified as a primitive neuroectodermal tumor of the central nervous system (CNS-PNET) [1]. They are known to be highly invasive embryonal neuro-epithelial tumors that metastasize via cerebrospinal fluid (CSF) and are often difficult to treat [2,3]. Chemotherapy is widely accepted as part of the multimodality treatment approach for MB. However, it is associated with debilitating toxicity and potential long term disabilities [4]. Overtreatment of MB patients with chemotherapeutic agents is attributable to long-term toxicities in majority of the cases [5]. Reduction in the doses of chemo-therapy with a more targeted approach will benefit these patients. Hence, to institute treatment modification, refinement of the existing clinical stratification system is imperative to further boost the outcome [6,7]. This can be achieved with improved understanding of the tumor biology to explore and apply the novel experimental therapeutics, especially for the one involving small molecule inhibitors. There are intriguing and accumulative evidences that promises the potential role of small molecules like NSAIDs (non-steroidal anti-inflammatory drugs) in cancer therapeutics [8]. Our previous studies have investigated the anti-cancer properties of Tolfenamic Acid (TA) in different types of cancers, including medulloblastoma [9–15]. TA is shown to induce growth inhibitory effect by suppressing survivin expression [16,17]. Survivin is associated with the progression and aggressiveness of the tumor [18–20]. Nevertheless, the use of such anti-inflammatory agent as a monotherapy will term

insufficient in absolute elimination of cancer [21]. Thus, the focus of this study was to investigate the use of a NSAID as an effective adjuvant for improving the outcome of conventional chemotherapy in childhood brain tumors. Multiple studies have illustrated three modes of action for demonstrating the adjuvant therapeutic efficacy of NSAIDs like chemo-sensitization, chemo-protection and modifications in chemo-drug pharmacokinetics or metabolism [8]. Consequently, in the current study we aimed to establish the probable mechanism leading to the enhanced combined anti-neoplastic effect of TA and chemotherapeutic drugs in MB cells. We tested the efficacy of conventional chemotherapeutic agents, VCR and Cis, in the current combination study, as both the drugs are responsible for treatment associated long-term adverse effects in MB patients [22,23].

We initiated our study by screening for the effective growth inhibitory dose, that is less than the individual IC_{50} values of TA, VCR and Cis in MB cells. We intended to achieve an enhanced anti-proliferative response of VCR or Cis when used in combination with TA. As determined by the cell viability data and CI (combination index) values, we could identify the effective dose of TA (10 µg/mL) and VCR (DAOY: 2 ng/mL and D283: 1 ng/mL) that induced time- and dose-dependent growth inhibition of MB cell lines with moderate synergism and negligible toxicity caused to human derived primary astrocyte cells. Both TA and VCR individually are known to induce apoptosis and cell cycle arrest in many types of tumors in a dose-dependent manner [9,13,24–27]. Likewise, the combination of low doses of TA and VCR caused increase in annexin-V positive cells along with upsurge of caspase 3/7 activity and c-PARP expression compared to their individual treatment. VCR is an anti-tubulin agent, which induces G_2/M arrest by disrupting the mitotic spindle integrity, eventually leading to activation of apoptotic pathway [28]. Nevertheless, we demonstrated an increase in number of cells arrested at G_2/M phase induced by combination treatment.

We suggested the role of survivin inhibition by TA towards this enhanced anti-proliferative effect [9], as survivin is known for its dual role in apoptosis inhibition by suppressing caspase 3/7 activity [29] and promoting cell cycle progression by stabilizing the microtubules of mitotic spindle apparatus [30,31]. The inhibition of survivin results in increased anti-tubulin efficacy of VCR induced even at its low dose, leading to G_2/M arrest and increased caspase 3/7 activity (Figure 4.5). This mechanism suggests the possible role of TA in sensitizing MB cells towards chemotherapeutic agent, VCR.

In conclusion, this study illustrated that the co-treatment of TA with low dose of vincristine synergistically and exclusively enhanced the inhibition of MB cell proliferation compared to the individual treatment, with suggestive role of survivin inhibition. Moreover, its anti-proliferative effect was accompanied by an increase in apoptosis and obstruction of cell cycle progression determined by their respective markers. Thus, we have provided evidences that represents this combination strategy as a potential novel targeted therapeutic approach specific for MB that would aid in minimizing the associated deleterious side effects. However, further elucidation of the mechanism resulting in the synergistic effect of the combination treatment in MB is highly warranted.

FUTURE DIRECTIONS

Demonstrating the sensitization of MB cells by TA towards VCR treatment.

Based on the results obtained for this study we theorize that TA co-treatment down-regulates survivin expression and sensitizes MB cells to be treated with non-toxic dose of VCR. Inhibition of survivin may result in disruption of mitotic spindle apparatus during metaphase of mitotic cell division by low dose of VCR (Figure 4.5). The disruption of tubulin

polymers of spindle assembly by combination dose of TA and VCR leading to the increased G_2/M phase arrest compared to the VCR treatment alone can be determined by immunofluorescence microscopy for alpha- and beta-tubulins and by in vitro microtubule polymerization assay. For immunofluorescence technique, we can simultaneously determine the expression of survivin via fluorescent tagging of survivin protein. This approach will help in confirming the role of survivin in stabilizing the mitotic spindle structure essential for cell cycle progression, that is affected by the TA and VCR combination treatment at low dose.

Test on primary cultures w.r.t. the molecular subtypes of MB:

As discussed earlier, MB is classified into four different molecular subtypes and the clinical outcome differ for each of these groups. Evaluating the effect of proposed combination treatment on these subtypes could be beneficial clinically in providing targeted and personalized treatment for patients with various subtypes of MB. Among the two MB cell lines tested in this study, DAOY cells are classified under SHH subtype based on PCA (principal component analysis) and hierarchical clustering analysis [32,33] and D283 are normally classified as Group 4 MB but may represent few molecular features of Group 3 subtype like the elevated expression of c-myc protein [34,35]. In addition, we could also determine the efficacy of the combined treatment on patient derived primary cell cultures specific to each subtype.

Xenograft studies:

As a suggestive goal for future pre-clinical studies, the overall specificity and efficacy of TA+VCR treatment must be tested on mouse xenograft models for MB. The human tumor

cell line derived xenograft models are extensively used for anticancer drug development studies along with elucidating the molecular mechanisms affected by the treatment [36]. Immunodeficient mouse are used to develop xenograft models by implanting human derived cancer cell lines or primary tumor cultures, either at ectopic or orthotopic sites. This *in vivo* study will help in determining the systemic response of the combination treatment for inhibiting MB tumor growth.

Pathway or meta-analysis:

The combination treatment tested in this study also caused decrease in the expression of cell cycle regulators cyclin A, B1 and CDK4/6. However, the current data is not sufficient to link the synergistic effect of the combination with the altered expression of these cell cycle protein kinases. Identification of the specific mechanism responsible for TA+VCR synergism could be the next goal of this study. Differential expression profiling using microarray analysis or top-down proteomics analysis via mass spectrometry could be beneficial in elucidating the various pathways affected by the combination treatment leading to cell cycle arrest and apoptosis. These methodologies could be useful in identifying other potential targets and epigenetic changes associated with the sensitizing effect of TA on VCR specific to MB cells, ensuring the reduced peripheral neurotoxicity of the chemo-therapeutic drug.

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