Thymosin-β4 is a determinant of drug sensitivity for Fenretinide and Vorinostat combination therapy in neuroblastoma

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ABSTRACT

Retinoids are an important component of neuroblastoma therapy at the stage of minimal residual disease, yet 40-50% of patients treated with 13-cis-retinoic acid (13-cis-RA) still relapse, indicating the need for more effective retinoid therapy. Vorinostat, or Suberoylanilide hydroxamic acid (SAHA), is a potent inhibitor of histone deacetylase (HDAC) classes I & II and has antitumor activity in vitro and in vivo. Fenretinide (4-HPR) is a synthetic retinoid which acts on cancer cells through both nuclear retinoid receptor and non-receptor mechanisms. In this study, we found that the combination of 4-HPR + SAHA exhibited potent cytotoxic effects on neuroblastoma cells, much more effective than 13-cis-RA + SAHA. The 4-HPR + SAHA combination induced caspase-dependent apoptosis through activation of caspase 3, reduced colony formation and cell migration in vitro, and tumorigenicity in vivo. The 4-HPR and SAHA combination significantly increased mRNA expression of thymosin-beta-4 (Tβ4) and decreased mRNA expression of retinoic acid receptor α (RARα). Importantly, the up-regulation of Tβ4 and down-regulation of RARα were both necessary for the 4-HPR + SAHA cytotoxic effect on neuroblastoma cells. Moreover, Tβ4 knockdown in neuroblastoma cells increased cell migration and blocked the effect of 4-HPR + SAHA on cell migration and focal adhesion formation. In primary human neuroblastoma tumor tissues, low expression of Tβ4 was associated with metastatic disease and predicted poor patient prognosis. Our findings demonstrate that Tβ4 is a novel therapeutic target in neuroblastoma, and that 4-HPR + SAHA is a potential therapy for the disease.
Keywords: Thymosin-β4 (Tβ4), 4-HPR, SAHA, neuroblastoma, Histone deacetylase inhibitors and retinoic acid receptor alpha (RARα).
1. Introduction

Neuroblastoma is the most common cancer in infants and accounts for 7% of all cancers in children (Barone et al., 2013). Neuroblastoma begins in embryonal neural crest cells which later give rise to the sympathetic nervous system, and is caused in part by factors which arrest differentiation (Calao and Sekyere, 2012). In the past decade, improved treatment of minimal residual disease by the addition of ch14.18 (a monoclonal antibody against the tumor-associated disialoganglioside GD2), GM-CSF and interleukin-2 to standard isotretinoin (13-cis-RA) therapy has increased survival rates (Yu et al., 2010). However, a considerable proportion of patients still experience relapse and are refractory to conventional treatment approaches.

Retinoids are vitamin A analogues required for normal morphogenesis and maintenance of diverse embryologic and adult tissues, which act on cells by binding nuclear receptors (Germain et al., 2002). Retinoids such as all-trans-retinoic acid (aRA), 13-cis-RA, and 4-HPR at low doses are capable of inducing differentiation and apoptosis in cancer cells (Schroeder et al., 2007). Neuroblastoma cells differentiate in response to retinoic acid in vitro, an observation that has led to clinical trials using the 13-cis-RA (Matthay et al., 2009). 4-HPR is a synthetic retinoid which works on cancer cells through nuclear receptor-dependent and -independent signaling mechanisms (Sogno et al., 2009). Non-receptor mechanisms of 4-HPR sensitivity include production of ceramide and reactive oxygen species (Maurer et al., 1999). 4-HPR has been used as a single agent in early phase clinical trials for neuroblastoma and ovarian cancer (Colombo et al., 2006; Villablanca et al., 2006). Clinical trials have revealed that 4-HPR
is a highly active therapeutic and chemo-preventive agent with minimal side-effects in neuroblastoma patients (Formelli et al., 2008). A phase I/II trial of oral 4-HPR in children with high-risk, relapsed solid tumors demonstrated minimal 4-HPR toxicity, but only stable disease as the best clinical response. However, newer oral 4-HPR preparations have shown markedly increased bio-availability (Villablanca et al., 2011). In addition, 4-HPR can act synergistically with other anti-cancer compounds (Fang et al., 2011; Shibina et al., 2013).

Increased histone deacetylase (HDAC) activity is a common causal factor in human cancer that leads to transcriptional silencing of tumor suppressor genes (Steele et al., 2009). HDAC inhibitors prevent these deacetylases removing acetyl groups from histone tails, thereby promoting gene transcription (Johnstone, 2002). Several HDAC inhibitors have entered early phase trials, and, Vorinostat (SAHA) has been approved for use in adult cutaneous T cell lymphoma (Duvic et al., 2007). The HDAC inhibitor side-effect profile is low when compared with cytotoxic chemotherapy (Hainsworth et al., 2011). However, early phase trials and pre-clinical data suggest that HDAC inhibitors will be most effective when used in combination with other anticancer agents (Liu et al., 2007). Synergistic anti-tumor activity between HDAC inhibitors and retinoids has been observed in a variety of preclinical models (Kato et al., 2007; Spiller et al., 2008). SAHA combined with 13-cis-RA, was well-tolerated in a Phase I/II pediatric trial, but the best response for relapsed solid tumor patients was stable disease (Fouladi et al., 2010). Two unbiased preclinical screens identified retinoid signal activation as the most effective method of augmenting the HDAC inhibitor anti-cancer signal (Epping et al., 2009; Epping et al., 2007).
In this study, we used human neuroblastoma cell lines, flow cytometry, gene-expression analyses, siRNA knockdown, gene expression profiling and a xenograft tumor model to evaluate the 4-HPR + SAHA combination treatment of neuroblastoma for therapeutic effects and identification of genes which mediated the treatment response. We showed the 4-HPR + SAHA combination has significant cytotoxicity against neuroblastoma cells and identified an actin regulatory protein, thymosin-beta-4 (Tβ4), as a critical molecular target of the combination therapy effects on neuroblastoma cell survival, cell migration and focal adhesion formation.

2. Materials and methods

2.1 Cell culture and reagents
Neuroblastoma cells, BE(2)-C (MYCN-amplified), SH-SY5Y (MYCN-non-amplified) and NBL-S (MYCN-non-amplified) were purchased from ATCC. They were cultured in DMEM medium (Gibco, Life Technologies) supplemented with 10% fetal calf serum. MRC-5 fibroblast cells were purchased from ATCC. These cells were cultured in alpha-MEM medium (Gibco, Life Technologies) supplemented with 10% fetal calf serum. All cell lines were authenticated by CellBank Australia (NSW, Australia). 4HPR, SAHA, and 13-cis-RA were all purchased from Sigma (Sigma-Aldrich). Antibody for pro-caspase 3 and activated-caspase 3 were purchased from Cell Signalling Technology (USA).

2.2 In vitro combination studies
BE(2)-C, SH-SY5Y, NBL-S and MRC-5 cells were selected for these studies. 4-HPR was combined with SAHA at a fixed ratio of concentrations 6:1, respectively. Cell
viability was measured using Alamar blue assay and the fluorescence output was measured on Victor 3™ plate reader (Perkin Elmer). The combination index (CI) was calculated based on the Chou–Talalay equation, which takes into account both potency ($Dm$ or IC50) and the shape of the dose–effect curve. (Chou and Talalay, 1984) CI<1, CI=1, CI>1 indicate synergism, additive effect and antagonism, respectively. CalcuSyn software (Biosoft, Ferguson, MO, USA) was used for the Chou–Talalay combination index analysis.

2.3 Flow cytometry

Neuroblastoma cell lines; BE(2)-C & SH-SY5Y and normal lung fibroblast; MRC5 were treated with 2uM 4-HPR and 0.33uM SAHA for 48 hours then fixed with 80% ethanol. Propidium iodide (PI, 10 ug/ml) (Sigma-Aldrich) and RNAse (5ug/ml) (Roche Applied Science) were added to each sample. Cell cycle and uptake of PI was analyzed on the FACSCalibur (BD Biosciences) and CellQuest™ software. Measurement of early stages of apoptosis was done using Annexin V-FITC conjugate (Molecular Probes, Life technologies) and 7AAD apoptosis detection kit (BD Pharmingen™) following manufacture protocols.

2.4 Caspase-3 activity assay

BE(2)-C cells were treated with DMSO (control), 2uM 4-HPR, 0.33uM SAHA and combination of SAHA+ 4HPR for 48 hours. Cytosolic lysates were extracted to measure caspase-3 activity using the Caspase-3 Colorimetric Assay Kit (Abcam). In brief, 150-200ug proteins were mix with 2x sample buffer and 200uM DEVD-pNA substrate. The
pNA light emission was read at 405nm on the Victor 3TM plate reader (Perkin Elmer) at regular one hour time intervals to detect the optimum light emission. Duplicate samples were also performed with the addition of 40uM caspase-3 inhibitor, Z-DEVD-FMK (R&D Systems), to the cytosolic lysate.

2.5 Colony forming assay

BE(2)-C cells were plated at 50 cells/well in Costar 6 Well Clear-TC treated plate a day prior to drug treatment to allow attachment. 2µM 4-HPR or 0.33 µM SAHA, or both reagents in combination were added to each well with growth medium, and incubated at 37°C, 5% CO2 for 12 days in dark. Growth medium was aspirated at the end of incubation and wash with PBS once. Cells were fixed and stained with 2–3 ml of a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 30 minutes. The staining was washed by immersing the plate in tap water and drying at room temperature overnight. Colonies with minimum of 20 cells were counted.

2.6 In vivo tumorigenic assay

The 4-HPR and SAHA anti-tumor efficacy studies were performed by GenScript (Piscataway, NJ 08854, USA) in a xenograft tumor mice model. 5x10^6 of BE(2)-C cells were subcutaneously injected into the right flank of a cohort of 32 female BALB/c nude mice. When the tumor size reached a volume of 54 mm³, the tumor-bearing nude mice were randomly assigned to 4 treatment groups: vehicle control, 4-HPR (1.45mg/kg i.v), SAHA (35mg/kg i.p) or 4-HPR + SAHA combination for 18 days (Hill et al., 2009; Reddy et al., 2004). Tumor volume was measured every 2 days with a caliper, and the volume
was expressed in mm$^3$ using the formula: Volume = $\frac{1}{2} \times A \times B^2$ where $A$ and $B$ are the long and short diameters of the tumor, respectively.

2.7 Differential gene expression and pathway analysis on microarray data

Microarray data was profiled using Affymetrix HuGene 1.0 st v1. BE(2)-C cells were treated with solvent control, 2µM of 4-HPR, 0.33µM of SAHA or 4HPR+SAHA for 24 hours. Differential gene analysis was conducted in R (http://www.rproject.org/). The raw gene expression data was loaded into R followed by analysis with packages from Bioconductor (http://www.bioconductor.org/). Using affy package (Gautier et al., 2004), the expression data were subjected to background correction and normalization with robust multi-array average measure (RMA). In three comparisons, (4-HPR vs. control, SAHA vs. control and 4-HPR + SAHA vs. control), the gene expression greater than 2 fold was considered to be differentially expressed. The pathway analysis was conducted with the differentially expressed genes against KEGG using DAVID (Huang da et al., 2009) and WebGestalt (Zhang et al., 2005).

2.8 RNA interference

Cells were reverse transfected with 40nM siRNA duplexes against Tβ4 and control siRNA (Dharmacon, Thermo Scientific) using LipofectamineTM 2000 (Invitrogen, Life Technologies) and Opti-MEM® (Gibco, Life Technologies) for 24 and 48 hours. Cells were then used in cell viability assays or their RNA was isolated for real-time PCR analysis.
2.9 **Real-Time PCR**

RNA from cell lines was synthesized into cDNA using SuperScript III reverse transcriptase (Invitrogen, Life Technologies) and Oligo (dT)18 (Bioline) according to manufacture protocols. Real-time PCR was performed using Power SYBR Green mix (Applied Biosystems, Life Technologies) and PCR was carried out on the ABI 7500 thermo cycler (Applied Biosystems). Relative gene expression data was analysed using the 2-ΔΔCT method. Forward sequences and reverse sequences: Tβ4: 5’ CAGAGACGCAAGAGAAAAATCCA 3’ and 5’ CGATTGCCTGCTTGCTT 3’. β2M: 5’ ACTGGTCTTTCTATCTCTTGCTACTACCTGA 3’ and 5’ TGATGCTGCTTACATGTCTCGAT 3’.

2.10 **Chromatin immuno-precipitation (ChIP) assays**

Standard ChIP assays were performed as described (Iraci et al., 2011). Antibodies used in this study were as follows: IgG (sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA), RARα (Santa Cruz Biotechnology). The primers used were as follows: Tβ4-A, 5’-ttagttattatgcactgtccccc-3’ (forward) and 5’-ccctccaacactcataacat-3’ (reverse); Tβ4-B, 5’-tcgaaataaaagaatgcctgctc-3’ (forward) and 5’-cttggggaccagtgcata-3’ (reverse); Tβ4-C, 5’-aatcacttaagggtgctttacgtc-3’ (forward) and 5’-tacgattcctgtgactc-3’ (reverse); Tβ4-intron A, 5’-cactgcagaccacagactt-3’ (forward) and 5’-ttagggaatgcgctcccagag-3’ (reverse); Tβ4-intron B, 5’-cagaccacagttcagcctc-3’ (forward) and 5’-ttggaatgcctc-3’ (reverse); Control (-3000bp), 5’-tagcctttcagttgctaa-3’ (forward) and 5’-ctggagcaactaatagact-3’ (reverse).
2.11  *In vitro wound healing assay*

BE(2)-C cells were plated in culture insert (Ibidi, Martinsried, Germany) and allowed to reach 90% confluency overnight. Treatment drugs (4-HPR, SAHA, or both) were added to the culture medium and incubated for 24 hours. The culture insert was then removed, leaving a uniform gap for cells to migrate toward. Cells were monitored and images were taken every hour with Zeiss Axiovert 200M live cell imager up to 13 hours. The wound was analyzed with AxioVision 4.8 software. Migration index (%) was computed as following, ("wound area at beginning" -wound area at 13 hours) / (wound area at beginning) x100%.

2.12  *Invasion assay*

Cell invasion assays were performed as previously described (Lees et al., 2011). Following gel polymerization, the media containing drug was added to the wells (0.75 µM 4-HPR and 0.125 µM SAHA alone or in combination). After incubation for 72 hours, the cultures were fixed with 4% paraformaldehyde and then stained with fluorescently-tagged phalloidin and nuclei counterstained with DAPI. Images of stained cells were captured using a Leica SP5 confocal scanning laser microscope (Leica Microsystems, Germany) with a 10 X air objective. Optical sections were captured in 3 µm steps beginning with the bottom layer of cells underneath the collagen gel. Maximum projections of z-stacks were generated using the Leica software, final micrograph images and grey level adjustments were prepared in Adobe Photoshop.

2.13  *Immunofluorescence for focal adhesions*
Immunofluorescence was carried out as described previously (Bell et al., 2013). Cells were grown on 8-well chamber slides prior to fixing with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with anti-paxillin (Abcam, Cambridge, UK) to visualize the focal adhesions. Secondary antibody staining with Alexa Fluor 555-conjugated anti-mouse antibody (Molecular Probes; Invitrogen) was applied. Phalloidin conjugated with Alexa Fluor 488 (Life Technologies) was applied to visualize the actin stress fibres. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to visualize nuclear material. Confocal microscopy images were acquired using an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan), ×40 objective lens, NA 1.30. A Z series of images of 0.44 μm/section was collected using sequential scanning. Images were processed using the Olympus FluoView software (Olympus).

2.14 Statistical analysis

All statistical tests were performed in GraphPad Prism5 (GraphPad Software Inc.) All experiments were performed in minimum of triplicates. Two-way ANOVA with Bonferroni post-test and the One-way ANOVA with Tukey’s post-test were performed for the statistical analysis. The two-sided student t-tests were used in the tumor volume analysis.

3. Results
3.1 4-HPR + SAHA combination therapy has significant cytotoxicity to neuroblastoma cells

We first examined the cytotoxic effect of 4-HPR + SAHA on three human neuroblastoma cell lines (BE(2)-C, SH-SY5Y and NBL-S) by Alamar blue assay at a range of different concentrations (4-HPR: 1.33µM to 4.5µM, and SAHA: 0.22µM to 0.75µM) using a 6:1 molar ratio of each compound (Figure 1A-C). To assess drug synergy the Combination Index (CI) values were calculated by CalcuSyn at the fixed ratio of the drug concentrations, which tested for the cytotoxicity assays. 4-HPR + SAHA showed slight to moderate synergism in BE(2)-C and NBL-S neuroblastoma cell lines, with a CI < 1 for three of the four drug concentrations tested. CI was 0.94 for BE(2)-C cells and 0.95 for NBL-S cells at 2µM 4-HPR + 0.33µM SAHA after 48 hours treatment. CI was 0.85 for BE(2)-C cells and 0.82 for NBL-S cells at 3µM 4-HPR + 0.5µM SAHA. CI was 0.47 for BE(2)-C cells and 0.86 for NBL-S cells at 4.5µM 4-HPR + 0.75µM SAHA for 48 hours. 4-HPR + SAHA showed an additive effect on treated SH-SY5Y cells at two concentrations after 48 hours treatment (CI = 1 for 3µM 4-HPR + 0.5µM SAHA and 4.5µM 4-HPR + 0.75µM SAHA) (Chou, 2006). SAHA and 4-HPR as single agents, or in combination, were minimally toxic to treated MRC-5 normal fibroblasts at concentrations up to 4.5 µM for 4-HPR and 0.75 µM for SAHA (Figure 1D). In contrast, the combination of 2µM 13-cis-RA and 0.33 µM SAHA exhibited no significant synergy or therapeutic toxicity in treated BE(2)-C cells (Figure 1E).

3.2 4-HPR + SAHA combination therapy induced caspase-dependent apoptosis through activation of caspase 3
Since both 4-HPR + SAHA can individually induce apoptosis in neuroblastoma cell lines (Armstrong et al., 2012; De los Santos et al., 2007), we examined the apoptotic effects of the combination in two neuroblastoma cell lines. We observed significantly higher levels of the sub-G1 apoptotic cell population in BE(2)-C and SH-SY5Y neuroblastoma cells when the two drugs were combined, in comparison with either drug alone (Figure 2A). The combination did not induce apoptosis in MRC-5 normal fibroblast cells using propidium iodide staining and flow cytometric analysis (Figure 2A).

We next examined the early and late stages of apoptosis, by treating neuroblastoma cells with either 2µM 4HPR or 0.33µM SAHA alone, 4-HPR + SAHA, or 13-cis-RA + SAHA for 48 hours, then stained with Annexin V-Fluorescein isothiocyanate (FITC) and 7-Amino Actinomycin D (7AAD). The 4-HPR + SAHA combination induced a marked increase in Annexin V positive neuroblastoma cells when compared with either drug alone, or a combination of 13-cis-RA + SAHA in both BE(2)-C and SH-SY5Y cells (Figure 2B and C). The percentage of early (Annexin V positive and 7AAD negative) and late apoptotic cells (both Annexin V and 7AAD positive) was significantly higher for the 4-HPR + SAHA combination (Supplementary Figure 1 and Supplementary Figure 2).

To investigate the mechanism of the 4-HPR + SAHA-induced apoptosis, BE(2)-C and SH-SY5Y cells were treated with either 2µM 4HPR or 0.33µM SAHA alone, or both reagents for 48 hours and then protein lysates were immunoblotted for the protein level of activated caspase 3. Treatment with 4-HPR + SAHA caused increased activated caspase-3 protein levels at 48 hours in both BE(2)-C and SH-SY5Y (Figure 2D and E). In addition, BE(2)-C cells were treated with DMSO control, 2µM 4-HPR, 0.33µM SAHA and combination of SAHA + 4-HPR for 48 hours. Cytosolic lysates were extracted to
measure the caspase-3 enzymatic activity using the Caspase-3 Colorimetric Assay Kit. The combination of 4-HPR and SAHA treatment also increased the level of caspase 3 enzymatic activity in comparison with the treatment of the two single agents, and this caspase 3 enzymatic activity was blocked by a caspase 3 inhibitor in BE(2)-C cells (Figure 2F).

3.3 4-HPR + SAHA combination therapy reduces colony formation in vitro and tumorigenicity in vivo

We next performed clonogenicity assays on BE(2)-C cells treated with 4-HPR + SAHA. Colonies in vitro were defined as cluster of 20 cells or more after 12 days (Franken et al., 2006). While colony formation was significantly reduced by 4-HPR or SAHA alone, combination therapy completely abrogated colony formation (Figure 3A). To determine whether 4-HPR + SAHA had a synergistic activity in vivo, we selected BE(2)-C, a cell line which exhibited strong synergy in vitro. While 4-HPR or SAHA alone reduced tumor volume by 30% of control at day 18 of treatment, combination therapy had a significantly greater effect, reducing tumor volume by 50% at day 18 (The control vs SAHA+4-HPR: P=0.0023, SAHA vs SAHA+4-HPR: P=0.0238 and 4-HPR vs SAHA+4-HPR: P=0.0486). (Figure 3B and Supplementary Table 1). During the experimental period, the mice in all treated groups showed no decrease in body weights (Supplementary Figure 3).

3.4 Actin binding protein, Tβ4, expression is induced by 4-HPR + SAHA treatment
To identify factors which regulated the sensitivity of neuroblastoma cells to 4-HPR + SAHA, we performed an Affymetrix microarray of transcriptomes in BE(2)-C cells treated with solvent control, 2μM 4-HPR, 0.33μM SAHA or the combination for 24 hours. Pathway analysis of differentially expressed genes against KEGG using DAVID (Huang da et al., 2009) and WebGestalt,(Zhang et al., 2005) showed a number of signaling pathways which were significantly altered by the combination treatment in comparison with untreated control and the single agents alone, such as, focal adhesion formation, cell adhesion molecules and regulation of actin cytoskeleton pathways from the KEGG database (Figure 4A and B).

One of the most significantly up-regulated genes by the combination was Tβ4 (TMSB4X). On the microarray Tβ4 was induced 2.9-fold by 4-HPR, 4.6-fold by SAHA, and 19.39-fold by the combination treatment, compared to untreated control (Figure 4C). Real-time RT-PCR analysis confirmed similar levels of Tβ4 induction (Figure 4D). Tβ4 is a polypeptide involved in regulation of the actin cytoskeleton and cell migration, which can demonstrate the characteristics of an oncogene or a tumor suppressor gene, dependent on the cellular context (Caers et al., 2010a; Cha et al., 2003). Thus, we next analysed publically available mRNA expression array datasets from primary human neuroblastoma tumour tissues for a relationship between Tβ4 expression level and patient prognosis (Kocak et al., 2013). When patient cohorts were subdivided around the median Tβ4 expression level, we found high Tβ4 expression strongly correlated with good patient prognosis in two separate cohorts of 102 and 477 neuroblastoma patients (Seeger dataset: p<0.0001; Kocak dataset: HR = 3.85, 95% CI = 2.38 - 6.23, p = 3.13e-09 for the overall survival and HR=2.01, 95% CI = 1.45 - 2.79, p = 2.01e-05 for the
event-free survival) (Figure 4E, F and G). Gene expression analysis for Tβ4 revealed that higher expression correlated with known indicators of good prognosis in neuroblastoma, including early clinical stage, MYCN non-amplification and younger patient age (Figure 4H).

The nuclear retinoid receptor and signaling molecule, retinoic acid receptor alpha (RARα), has been shown to be a resistance factor for SAHA effects on cancer cells (Epping et al., 2007). Indeed, in its unliganded state, RARα acts as a transcriptional trans-repressor. Thus, we examined the hypotheses that RARα was a resistance factor for 4-HPR + SAHA in neuroblastoma cells and a repressor of Tβ4 transcription. The expression of RARα was significantly repressed by 4-HPR + SAHA in treated BE(2)-C and SH-SY5Y cells compared with 4-HPR alone and with SAHA alone, whereas 13-cis-RA + SAHA had no effect on RARα (Figure 5A). Transient transfection of two RARα-specific siRNAs reduced cell viability in control, single agent or combination treated neuroblastoma cells (Figure 5B). In contrast, RARα overexpression blocked the effect of combination therapy on cell viability (Figure 5C). RARα protein expression was decreased by single agent and combination treatment (Supplementary Figure 4). Moreover, knockdown of RARα by two RARα-specific siRNAs significantly induced Tβ4 expression and the knockdown of RARα mRNA and protein were confirmed by RT-PCR and western blot. (Figure 5D). However, we were unable to demonstrate direct binding of the RARα protein to the Tβ4 gene promoter using ChIP assay in the absence and presence of the combination treatment, whereas RARα directly bound the RARβ promoter region in the assay, as has been reported (Supplementary Figure 5 and data not shown) (Wang et al., 2010). These data suggested RARα indirectly repressed Tβ4
expression in neuroblastoma cells and was itself a repression target of 4-HPR + SAHA treatment.

3.5 Up-regulation of Tβ4 is necessary for the 4-HPR + SAHA effects on cell viability, cell migration and focal adhesion formation

To determine whether induction of Tβ4 expression in neuroblastoma cells enhanced the cytotoxic effect of 4-HPR + SAHA, we transiently transfected neuroblastoma cells with siRNAs targeting Tβ4 for 24 hours, then treated cells with single agents or the combination for 48 hours. Neuroblastoma cells transfected with the two Tβ4 siRNAs exhibited a marked resistance to the cytotoxic effects of the 4-HPR + SAHA in both BE(2)-C and SH-SY5Y cells (Figure 6A and B).

Since Tβ4 regulates the cytoskeleton, we next assessed the role of combination therapy and Tβ4 on neuroblastoma cell migration using in vitro scratch-wound assays on confluent monolayers (Marshall et al., 2010). BE(2)-C and SH-SY5Y cells treated with 4-HPR + SAHA migrated slower than the single agents or untreated control at 12 hours from the time of wounding (Figure 6C and Supplementary Figure 6). In contrast, 13-cis-RA + SAHA treated cells did not reduce cell migration in comparison with control or single agent (Figure 6C and Supplementary Figure 6). An in vitro collagen gel invasion assay showed that 4-HPR and SAHA alone were as effective as the combination in reducing invasion of BE(2)-C cells (Figure 6D and E), indicating 4-HPR+SAHA has stronger effect in decreasing cell migration, but not invasion, in comparison with single agents. Furthermore, we found that knockdown of Tβ4 increased migration of untreated and 4-HPR+SAHA treated neuroblastoma cells,
suggesting Tβ4 expression is required for 4-HPR+SAHA reduced cell migration in neuroblastoma cells (Figure 6F and Supplementary Figure 7). Since Tβ4 affects actin function and the actin cytoskeleton during focal adhesion formation (Caers et al., 2010a; Wozniak et al., 2004), we next investigated whether Tβ4 plays a role in focal adhesion formation in the combination treatment. We showed that 4-HPR + SAHA markedly reduced actin filaments in neuroblastoma cells stained with the high-affinity filamentous actin (F-actin) probe, phalloidin (Figure 6G). Focal adhesions are coupled to bundles of polymerized actin filaments or stress fibres. The disassembly of stress fibres is accompanied by a breakdown of the focal adhesion tethered at the end of the actin filament bundle. When we treated neuroblastoma cells with 4-HPR + SAHA, we found focal adhesion formation was reduced 3-fold by SAHA alone or 4-HPR + SAHA treatment, compared with 4-HPR or solvent control alone (Figure 6H). However, the cells treated with SAHA alone still maintained similar cell shapes as untreated cells, but we have observed some changes in cell shape in the combination treated cells, which may due to the effect of the combination treatment on the structure of actin filaments (Figure 6G). Most importantly, knockdown of Tβ4 expression completely reversed the 4-HPR + SAHA treatment effect on focal adhesion formation and was also accompanied by reversal of the cell shape effects in the 4-HPR + SAHA treated cells (Figure 6H), indicating Tβ4 expression was necessary for the effect of 4-HPR + SAHA on focal adhesion formation.

4. Discussion
Preclinical studies suggested that combination of HDAC inhibitor and retinoid therapy would prove to be an effective anticancer treatment in the clinic, however, this promise
has not been reflected in early phase trials. Here we provide evidence of a novel and potent combination between a synthetic retinoid, 4-HPR, and a HDAC inhibitor, SAHA, which was far more effective than the retinoid in current clinical use in neuroblastoma, either 13-cis-RA alone, or in combination with SAHA. The molecular mechanisms underlying the cytotoxic action studies has identified a novel anticancer treatment target, Tβ4, a regulator of the actin cytoskeleton, and consequent inhibitory effects on cell migration and focal adhesion formation. Taken together, our data suggest a unique 4-HPR+SAHA treatment effect on up-regulation of Tβ4, inhibition of focal adhesions and cell migration, with important implications for the prevention of metastasis in patients with minimal residual neuroblastoma (see the schematic overview in Figure 7).

In the neuroblastoma cell lines and drug concentrations tested in this study, we found that 4-HPR and SAHA alone had a modest effect on cell viability and apoptosis in BE(2)-C cells, whereas 4-HPR and SAHA alone had no effect on apoptosis in SH-SY5Y cells. However, in both cell lines, the combination of the two drugs was significantly induced apoptosis and reduced cell viability. We observed that the combination of these two agents had minimal toxic effects on normal human fibroblasts in vitro. The combination of 4-HPR + SAHA increased activated caspase-3, indicating that the cytotoxic effects of this drug combination may act via a caspase-dependent apoptotic pathway. However, 4-HPR has been reported to be cytotoxic agent and 13-cis-RA is a differentiating agent for neuroblastoma cell lines (Ponzoni et al., 1995). In this current study, we did not perform experiments to determine the role of 4-HPR in differentiation in neuroblastoma cells. In addition, the 13-cis-RA reduces cell proliferation after one week of treatment, therefore, therefore, more studies are required for the comparison of
in vitro and in vivo drug efficacy between 4-HPR + SAHA and 13-cis-RA + SAHA combinations, especially in the induction of cell differentiation in neuroblastoma.

Tβ4 forms a 1:1 complex with G (globular) actin, sequestering G-actin monomers from polymerizing to form F (filamentous) actin which comprise the cellular microfilaments needed for the normal cellular cytoskeleton and motility (Carlier et al., 1996; Shum et al., 2011). Different studies indicated a pivotal role of Tβ4 in the metastatic process of solid tumors. Up-regulation of Tβ4 was found in Ras-transformed fibroblasts and metastatic human melanoma cells (Nummela et al., 2006). Overexpression of the Tβ4 gene was associated with increased invasion of SW480 colon carcinoma cells and the distant metastasis of human colorectal carcinoma (Wang et al., 2004). Our study suggests the role of Tβ4 in motility of cancer cells is context dependent, since we found that low levels of Tβ4 correlated with advanced clinical stage in vivo and migration in vitro of neuroblastoma cells. Cell migration is initiated by protrusion at the leading edge of the cell, the formation of peripheral focal adhesions, the exertion of force on these adhesions, and finally the release of the adhesions at the rear of the cell (Tilghman et al., 2005). Treatment with exogenous Tβ4 resulted in the formation of smaller focal adhesions in human conjunctival cells (Sosne et al., 2002). We hypothesize that Tβ4 may play a role in the inhibitory effects of 4-HPR + SAHA on focal adhesion formation in neuroblastoma cells. Indeed, we found that Tβ4 knockdown completely reversed the 4-HPR and SAHA treatment effect on focal adhesion formation, suggesting that 4-HPR and SAHA treatment alters the actin cytoskeleton in a Tβ4-dependent manner in neuroblastoma cells.
While some studies have suggested that Tβ4 has a role in metastasis (Wang et al., 2004) (Xiao et al., 2012), Caers et al have shown a tumor suppressive function of Tβ4 in myeloma development (Caers et al., 2010a). Tβ4 is a poor prognostic factor in a number of different types of tumors and a good prognostic indicator in multiple myeloma (Caers et al., 2010a; Xiao et al., 2012) (Caers et al., 2010b). Our finding indicated that the neuroblastoma patients with high expression of Tβ4 not only have longer event-free survival, but other good prognostic indicators in neuroblastoma, such as early clinical stage, MYCN non-amplification and younger patient age were also associated with high Tβ4 levels. The mechanistic explanation of this discrepancy is not clear, and the functional role of Tβ4 in neuroblastoma is currently unknown. Our data showed that up-regulation of Tβ4 was required for the 4-HPR + SAHA effects on cell viability, and down-regulation of Tβ4 significantly increased cell migration, suggesting that induction of Tβ4 limits neuroblastoma cell migration and leads to death of neuroblastoma cells. The explanation for the different functions of Tβ4 among cancers of different tissue origins may relate to tissue of origin-specific differences in the regulation of cytoskeleton, but will require further investigation.

RARα is a nuclear hormone receptor and dual-function transcription factor, which is bound to co-repressor complexes in the unliganded state, but forms heterodimers with other retinoid receptors and can transactivate its target genes, upon retinoid ligand binding (Glass and Rosenfeld, 2000). RARα, and, preferentially expressed antigen of melanoma, both repressor proteins for the retinoid signal, were shown to mediate resistance to HDAC inhibitors (Epping et al., 2007). Furthermore, RARα-deficient cells showed enhanced sensitivity to HDAC inhibitors in vitro and in vivo (Epping et al.,
Our data indicates that RARα acted as a resistance factor for the anti-cancer effect of SAHA and 4-HPR, and down-regulation of RARα potently reverses this inhibitory effect, allowing an increase in Tβ4 and reducing cell viability. We have found a consensus retinoic acid response element in the promoter of Tβ4 gene, which matches the preferred binding site of RARα (Popperl and Featherstone, 1993). However, we were unable to demonstrate that direct binding of RARα to the Tβ4 gene promoter by chromatin immuno-precipitation. Future studies are required to determine whether RARα decreases Tβ4 mRNA expression through post-transcriptional events involving mRNA, or non-coding RNA-mediated post-transcriptional control (Belasco, 2010).

Our in vitro results show that 4-HPR+SAHA therapy exhibited high levels of synergy at low concentrations, however, our single in vivo murine xenograft experiment using BE(2)-C cells showed only an additive therapeutic effect of the two agents. Indeed, 13-cis-retinoic acid was rejected as a treatment in neuroblastoma patients, despite a wealth of in vitro evidence of its therapeutic potential, when in vivo experiments and an early phase single agent human trial showed little effect against large solid tumor masses (Reynolds and Reynolds, 2004). However, subsequent randomized clinical trials demonstrated that 13-cis-retinoic acid was effective in preventing disease relapse at the stage of minimal residual neuroblastoma (Matthay et al., 2009). Furthermore, another study found that retinoic-acid-resistant neuroblastoma cell lines show altered MYC regulation and high sensitivity to 4-HPR, suggesting that therapy with 4-HPR may be especially effective if used against minimal residual neuroblastoma (Reynolds et al., 2000). Therefore, we hypothesize that 4-HPR+SAHA therapy may be also more effective against minimal residual neuroblastoma. However, to determine the effects of
4-HPR+SAHA on minimal residual neuroblastoma and metastasis *in vivo*, future studies will need to be performed in the mouse models of minimal residual neuroblastoma and metastases. The concentrations of 4-HPR and SAHA used in this study were within the range achieved in children in clinical trials (Fouladi et al., 2010; Villablanca et al., 2011). Since both 4-HPR and SAHA are in clinical use and have relatively low toxicity profiles, this combination should be assessed in a clinical trial setting in the future.

**Conflict of interest**

The authors declare no conflict of interest.

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**Appendix A**

**Supplementary material**

**REFERENCES**


PML/RARalpha targets promoter regions containing PU.1 consensus and RARE half sites in acute promyelocytic leukemia. Cancer Cell 17, 186-197.

Figure legends:

Figure 1 – The 4-HPR+SAHA combination demonstrates strong cytotoxicity on neuroblastoma cells with limited toxicity to normal MRC-5 fibroblasts. (A - D) BE(2)-C, SH-SY5Y, NBL-S neuroblastoma and MRC-5 lung fibroblast cells were treated with either 4-HPR, or SAHA, or the combination of both, at a range of concentrations for 48 hours, with the cell viability measured by Alamar blue assay. Statistical significance: Two-way ANOVA with Bonferroni post-test was performed. *p<0.05, **p<0.01, ***p<0.001. (E) BE(2)-C cells were treated with 2µM13-cis-RA, or 0.33µM SAHA, or the combination of both for 48 hours, before performing Alamar blue assay. N.S = not statistically significant.
Figure 2  – The combination of 4-HPR and SAHA induced caspase-dependent apoptosis through activation of Caspase 3. (A) BE(2)-C, SH-SY5Y and MRC5 cells were treated with either 2μM 4HPR, or 0.33µM SAHA alone, or both reagents for 48 hours. The cells were stained with propidium iodide (PI), and the percentage of cell population in sub-G1, G1, S and G2 phases were analyzed on the FACS Calibur. (B-C) BE(2)-C cells and SH-SY5Y cells were treated with either 2μM 4HPR or 0.33µM SAHA alone, 4HPR+SAHA or 2µM of 13-cis-RA+ 0.33µM of SAHA combinations for 48 hours then stained with annexin V-Fluorescein isothiocyanate (FITC) and 7-Amino Actinomycin D (7AAD), and the proportion of Annexin V positive cells were analysed on the FACS Calibur. Statistical significance: One-way ANOVA with Tukey’s post-test. *p<0.05, **p<0.005, ***p<0.0001. The “n.s” for no statistical significance. (D) The BE(2)-C and SH-SY5Y cells were treated with either 2μM 4HPR or 0.33µM SAHA alone, or both reagents for 48 hours. The level of activated Caspase 3 protein was determined from cytosolic protein lysates by Western blot. Pro-caspase 3 and activated caspase 3 antibodies were used to detect changes in caspase 3 protein expression. (E) Quantification of western blotting with an anti-Caspase 3 antibody by densitometry. (F) The BE(2)-C cells were treated with either 2μM 4HPR or 0.33µM SAHA alone, or both reagents for 48 hours in the absence or presence of a caspase 3 inhibitor, Z-VAD-fmk (40µM). The caspase 3 activity was measured by a colorimetric caspase 3 activity assay. The One-way ANOVA with Tukey’s post-test was used for the statistical analysis.
Figure 3 – The 4-HPR and SAHA combination blocked colony formation and reduced tumour growth of mouse xenograft. (A) The clonogenicity of BE(2)-C cells was determined using soft agar. The number of colonies were compared between untreated control, 2µM 4HPR or 0.33 µM SAHA, or both reagents treated BE(2)-C cells for 14 days. Statistical significance: One-way ANOVA with Tukey’s post-test. *p<0.001, ***p<0.0001. (B) The tumour volume of BE(2)-C cells injected into Balb/c nude mice and treated with respective drugs for 18 days. Each value shown in the figure represented data from 8 mice. Student T-test was performed to analyze tumor volume at day 18: vehicle control vs SAHA+4-HPR: P=0.0023; SAHA vs SAHA+4-HPR: P=0.0238 and 4-HPR vs SAHA+4-HPR: P=0.0486.

Figure 4 – The Tβ4 gene expression is modulated by the combination treatment and the high level of Tβ4 expression is associated with good prognosis in neuroblastoma patients. (A) A heatmap of top 60 differentially expressed genes comparing 4-HPR + SAHA combination treatment with untreated control are showed. The expressions of these genes in 4-HPR and SAHA as single agents are also showed in the heatmap. A number of genes up-regulated in the 4-HPR + SAHA combination treatment which associated with the pathways were indicated by arrows. (B) The top 15 pathways enriched by GSEA. GSEA revealed that pathways including focal adhesion, cell adhesion molecules, and regulation of action cytoskeleton are enriched by comparing the 4-HPR+SAHA combination versus solvent treated cells. The C2 collection of the Molecular Signatures Database v3.0 was queried for the enrichment. The table lists gene sets enrichment score (ES), normalised enrichment score (NES) and the relevant
nominal p-value (NOM p-val), with false discovery rate (FDR q-val) less than 0.25, and familywise-error rate (FWER p-val) less than 0.05. (C) The fold changes of Tβ4 gene expression in BE(2)-C cells with 4-HPR alone, SAHA alone or 4-HPR + SAHA for 24 hours in comparison with untreated control in the microarray study. (D) The mRNA expression of Tβ4 was analyzed by RT-qPCR following BE(2)-C cells treated with respective drugs for 24 hours. Statistical significance: ***p≤0.009. (E) Kaplan–Meier curve showed the probability of overall survival of patients according to the level of Tβ4 expression in the 102 neuroblastoma patients in the Seeger dataset (Sourced from publically available Oncogenomics – Seeger database). (F - G) Kaplan–Meier curves showed the probability of overall survival and Event-free survival of patients according to the level of Tβ4 expression in the 477 neuroblastoma patients in the Kocak dataset (R2 microarray analysis and visualization platform, http://r2.amc.nl). CI = confidence interval; HR = hazard ratio. P value was obtained from two-sided log-rank test. (H) expression of Tβ4 was evaluated in tumours from 477 neuroblastoma patients and dichotomized by known clinical indicators: stage (INSS 1,2,4S vs Stage 3,4), MYCN amplification status (non-amplified vs amplified) and age (<18 months vs >18 months).

Figure 5 – Down-regulation of RARα enhances effect of 4-HPR and SAHA combination and up-regulates Tβ4 mRNA expression. (A) The mRNA expression of RARα was analyzed by RT-qPCR following BE(2)-C and SH-SY5Y cells treated with respective drugs for 48 hours. Statistical significance: **p<0.005 and ***p<0.0005. (B) The BE(2)-C cells were transfected with scrambled siRNA control or two RARα specific siRNAs and treated with respective drugs for 48 hours, and the cell viability were analyzed by
Alamar Blue assay. (C) The BE(2)-C cells were stably transfected with empty vector or MEP-RARα expression vector and treated with either 2μM 4HPR, or 0.33μM SAHA or both reagents for 48 hours. The cell viability was analyzed by Alamar Blue assay. Statistical significance: **p<0.01, ***p<0.001. (D) The BE(2)-C cells were transfected with scrambled siRNA control or two RARα specific siRNAs for 96 hours, and the mRNA expression of RARα and Tβ4 were analyzed by RT-qPCR. The protein expression of RARα was analyzed by western blot. Statistical significance between RARα 1 and RARα 2 siRNAs knockdown compared to siRNA control: ***p<0.001, **p<0.01 and *p<0.05.

Figure 6 – Up-regulation of Tβ4 is necessary for 4-HPR and SAHA reduced cell viability and focal adhesion formation. (A) BE(2)-C and SH-SY5Y cells were transfected with control siRNA or two Tβ4 specific siRNAs for 24 hours then treated with respective drugs for 48 hours, and the cell viability was analyzed by Alamar Blue assay. Statistical significance difference between control siRNA and Tβ4 siRNA: *p<0.05, **p<0.01, ***p<0.001. (B) Knockdown of Tβ4 was confirmed with RT-qPCR analysis for Tβ4 mRNA expression BE(2)-C and SH-SY5Y cells. Statistical significance difference between control siRNA and Tβ4 siRNA: *p<0.05 and **p<0.01. (C) The histogram displays the proportion of wound closure over 12 hours from the time of wounding for BE(2)-C treated with respective drugs (solvent control, 0.75μM of 4HPR + 0.125μM of SAHA, 0.75μM of 13-cis-RA or 0.75μM of 13-cis-RA + 0.125μM of SAHA combination) for 24 hours. Migration index (%) = (“starting wound area” - “ending wound area”) and divided by “starting wound area”. Statistical
significance: *p<0.05 and **p<0.01. (D) Invasion of cells under the indicated conditions into 3D collagen gels. After invasion, cells were fixed and immunostained with phalloidin and DAPI. Cells were then analyzed by confocal microscopy, with images taken every 3µm. Arrow (scale bar) indicates direction of invasion. (E) Percentage of cell invasion (positive pixels at 36µm divided by total positive pixels at 0µm). Bars show the average of 5 separate experiments and error bars show the SEM. *p<0.05, One-way ANOVA with Tukey’s post-test. (F) The histogram displays the proportion of wound closure over 12 hours from the time of wounding for BE(2)-C transfected with control siRNA or Tβ4 specific siRNAs for 24 hours then treated with respective drugs for 24 hours (DMSO solvent control; and 0.75µM of 4HPR + 0.125µM of SAHA). P<0.05. Statistical analysis: Two-way ANOVA, Dunnett post-test. , *p<0.05, n = 3. (G) BE(2)-C cells were treated with 4-HPR, SAHA, or combination of both drugs for 24hr. Cells were then fixed and stained with anti-Paxillin (top row). Merged images were shown at the bottom row, with Paxillin represented in red, phalloidin represented in green, and DAPI in blue. The numbers of focal adhesion observed per µm² of cell area were quantitated using Axiovision (Carl Zeiss). (H) The quantification of the focal adhesion formation in BE(2)-C cells transfected with either control siRNA or Tβ4 siRNA for 24 hours and treated with respective drugs for an additional 24 hours. Error bars show the SEM, **p<0.01, *p<0.05, One-way ANOVA with Tukey’s post-test.

Figure 7 – Schematic overview of the signaling events that induced by 4-HPR+SAHA in neuroblastoma cells. The mechanism of synergy was dependent on repression of RARα expression, and induction of caspase 3 and Tβ4. Treated neuroblastoma cells exhibited
reduced cell viability, cell migration and increased apoptosis, in a Tβ4-dependent manner.