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Original Paper

Targeting the Difficult-to-Drug CD71 and **MYCN with Gambogic Acid and Vorinostat** in a Class of Neuroblastomas

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Key Words

CD71 (transferrin receptor 1) • MYCN • miR183 • Combination therapy • Vorinostat • Gambogic acid

Abstract

Background/Aims: Although neuroblastoma is a heterogeneous cancer, a substantial portion overexpresses CD71 (transferrin receptor 1) and MYCN. This study provides a mechanistically driven rationale for a combination therapy targeting neuroblastomas that doubly overexpress or have amplified CD71 and MYCN. For this subset, CD71 was targeted by its natural ligand, gambogic acid (GA), and MYCN was targeted with an HDAC inhibitor, vorinostat. A combination of GA and vorinostat was then tested for efficacy in cancer and non-cancer cells. *Methods:* Microarray analysis of cohorts of neuroblastoma patients indicated a subset of neuroblastomas overexpressing both CD71 and MYCN. The viability with proliferation changes were measured by MTT and colony formation assays in neuroblastoma cells. Transfection with CD71 or MYCN along with quantitative real-time polymerase chain reaction (gRT-PCR) and Western blotting were used to detect expression changes. For pathway analysis, gene ontology (GO) and Protein-protein interaction analyses were performed to evaluate the potential mechanisms of GA and vorinostat in treated cells. *Results:* For both GA and vorinostat, their pathways were explored for specificity and dependence on their targets for efficacy. For GA-treated cells, the viability/proliferation loss due to GA was dependent on the expression of CD71 and involved activation of caspase-3 and degradation of EGFR. It relied on the JNK-IRE1-mTORC1 pathway. The drug vorinostat also reduced cell viability/proliferation in the treated cells and this was dependent on the presence of MYCN as MYCN siRNA transfection led to a blunting of vorinostat efficacy and conversely, MYCN overexpression improved the vorinostat potency in those cells. Vorinostat inhibition of MYCN led to an increase of the pro-apoptotic miR183 levels and this, in turn, reduced the viability/proliferation of these cells. The combination treatment with GA and vorinostat synergistically reduced cell survival in the MYCN and CD71

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overexpressing tumor cells. The same treatment had no effect or minimal effect on HEK293 and HEF cells used as models of non-cancer cells. **Conclusion:** A combination therapy with GA and vorinostat may be suitable for MYCN and CD71 overexpressing neuroblastomas.

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Introduction

Neuroblastoma is the most common intra-abdominal malignancy of infancy [1] and it is the third most prevalent malignancy in children [2]. Despite improvements in treatment protocols, the patient outcome still depends on various genetic parameters that collectively determine the aggressiveness of the tumor. The gene expression profiles of various neuroblastoma samples include overexpression and amplification of certain genes [3, 4]; these include tumors having both transferrin receptor-1 (CD71) and MYCN amplified and overexpressed and displaying increased metastatic potential and tumor aggressiveness [5] (Fig. 1A-D). For such cancers, therapies that target both CD71 and MYCN may prove effective if indeed they are the drivers of the tumors. Both CD71 and MYCN have been demonstrated to be difficult to target in cancer cells [6, 7].

For cancer cells, the membrane receptor CD71 overexpression is known to increase cell proliferation and malignancy by enhancing and promoting iron import [6-8]. As such, for cancer types that include amplification/overexpression of CD71 such as thyroid carcinoma, breast cancer, non-small cell lung cancer, T lymphoblastic leukemia/lymphoma and neuroblastoma [9–12], CD71 may be a therapeutic target. As CD71 is the major iron importer to the cell, iron chelation therapy may also be effective for certain cancer types in inducing differentiation and reducing aggressiveness such as shown in neuroblastoma tumor models [13–17]. With respect to the MAPK/ERK pathway and CD71, iron levels indeed influence the activity of neuroblastoma, head, and neck squamous carcinoma and



Fig. 1. MYCN and CD71 expression in INSS stage 4 neuroblastoma patients. Scatter plots are shown for expression of MYCN and CD71 in neuroblastoma patient cohorts. The R2 platform was used for the calculation, and the AMC cohort (n=88) "Neuroblastoma public—Versteeg –88" (A) as well as the Cologne cohort (n=649; also subdivided to cohorts with various INSS stages) "Neuroblastoma—Kocak –649" (B-F). (G-H) Kaplan-Meier curves were generated from the survival data for neuroblastoma patients with tumor samples quantified for MYCN and CD71 expression. The data were from the cited cancer databases according to Materials and Methods.

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hepatocellular carcinoma cells [8, 20, 21]. Muñoz et al. [22] also showed the dependency for iron in activation of MAPK/ERK for maintaining plasticity, growth, and proliferation of neural-crest origin PC12 cells. Upon iron depletion, there is also activation of JNK1/2 and p38 that results in cell cycle arrest and apoptosis [23]. As such, iron chelation therapy, which is already available for treating iron overload in non-cancer indications, may be a potential treatment strategy for such tumors.

Gambogic acid (GA), a known ligand of CD71, has been shown to activate the apoptotic caspase-dependent pathway in human SH-SY5Y neuroblastoma cells [23]. Research has pointed to the activation of $TNF\alpha$ pathways and the capability of GA in suppressing proteasome activity as being responsible for apoptosis induction in target cells [24, 25]. Given the property of GA in binding and gaining entry via CD71 [26], GA could be a novel means of targeting CD71 overexpressing cancer types and merits a closer examination of its effects with respect to the expression of CD71 in target cells.

In neuroblastoma, amplification of *MYCN* oncogene is also relatively common and is thought to be responsible for activation of various anti-apoptotic genes that correlate with poor prognosis in patients [27–29]. MYCN has been shown to activate histone deacetylases (HDACs) and to silence various tumor suppressor genes by recruitment of DNA methyltransferases [29] and there are approved HDAC inhibitor drugs currently in the clinic for various cancer types [30, 31]. Treatment with histone deacetylase inhibitors (HDACi) has been shown to downregulate c-Myc expression at the transcription level and lead to acetylation of c-Myc at lysine 323, eventually leading to TRAIL activation and apoptosis [32]. HDACi- Trichostatin A (TSA)-treatment has also been shown to downregulate MYCN expression in neuroblastoma cells [33].

For neuroblastoma, as a high degree of clonal heterogeneity and intracellular signaling complexity exists [34–36], employing combinations of targeting agents may improve therapy outcome. In this study, we tested a combination of CD71 ligand GA with HDAC inhibitor vorinostat on candidate MYCN expressing neuroblastoma cells for viability and proliferation changes. In these cells, CD71-dependence of GA action was demonstrated as GA reduced CD71 surface expression in the treated cells and dependence of GA action on CD71 was seen as CD71 siRNA reducing the efficacy of GA. Treatment with GA also led to the degradation of EGFR, a major growth factor receptor in these cells. Separately, HDAC inhibitor vorinostat reduced MYCN expression and reduced cell viability and proliferation in the neuroblastoma cells tested. The combination of GA with vorinostat was effective in MYCN-CD71 positive neuroblastoma cells and was synergistic for viability/proliferation loss in the MYCN high-expressers cells.

Materials and Methods

Materials

Gambogic acid (GA) (PubChem CID: 9852185) and vorinostat (PubChem CID: 5311) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Panobinostat (PubChem CID: 6918837) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Desferoxamine (DFO), Salubrinal (sal) and rhodamine-123 were also from Sigma-Aldrich. Z-DEVD-FMK was from Santa Cruz Biotechnology (Dallas, TX, USA). Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), trypsin, MitoTracker Red and RNAlater reagents were from Thermo Fisher (Waltham, MA, USA). Rapamycin and SP600125 were purchased from Tocris (Bristol, UK). The pcDNA3.2/DEST/hTfR-HA was a gift from Robin Shaw (Addgene plasmid # 69610) and the pCDNA3-HA-human MYCN was a gift from Martine Roussel (Addgene plasmid # 74163). Antibodies against caspase-9, cleaved caspase-3, S6K, p-S6K (phospho-p70 S6 kinase Thr421/Ser424), ERK1/2, p-ERK1/2 (phospho-p44/42 MAPK Thr202/Tyr204), JNK, p-JNK (phospho-SAPK/JNK Thr183/Tyr185), CD71, MYCN, EGFR, p-EGFR (Ser1046/1047), p-EGFR (Tyr1068), p-mTOR (Ser2448), p-mTOR (Ser2481), mTOR, CHOP, IRE1, Ero1, acetyl-histone H3, p-Akt (Ser473), p-Akt (Thr308), Akt, p-PI3K (Tyr458), PI3K and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies of rabbit and mice origin were also from Cell Signaling Technology.

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conjugated secondary antibodies and Phalloidin Red were purchased from Thermo Fisher Scientific (Waltham, MA, USA). MYCN siRNA (h), HDAC2 siRNA and CD71 siRNA (h) were a pool of 3 target-specific 19-25 nt siRNAs designed to knock down gene expression (Product numbers: MYCN siRNA (h): sc-36003; CD71 siRNA (h): sc-37070; HDAC2 siRNA (h): sc-29345) and were

Table 1. List of primers for qPCR

Primer assay	Detected transcript(s)	Vendor	Cat. No
Human MYCN	NM_005378 (2736 bp)	Qiagen	PPH00199A
Human CD71	NM_001128148 (5100 bp)	Qiagen	PPH00990H
Human GAPDH	NM_001256799 (1455 bp)	Qiagen	PPH00150F
Human miR183	MIMAT0000261	Qiagen	MS00031507

purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The RNA extraction and cDNA preparation and qPCR kit, Syn-hsa-miR183 miScript miRNA Mimic, anti-hsa-miR183 miScript miRNA inhibitor, and primers for MYCN, CD71, GAPDH, and miR183 were purchased from Qiagen (Hilden, Germany) (Table 1). All the other reagents were of analytical grade or of the highest available purity.

Gene expression dataset analysis

R2 (R2: microarray analysis and visualization platform; http://r2.amc.nl (Department of Oncogenomics in the Academic Medical Center)) was used to investigate MYCN and CD71 expression in publicly available cohorts of primary neuroblastoma patients from German Neuroblastoma cohort (Neuroblastoma—Kocak -649, https://hgserver1.amc.nl) and Academic Medical Center (AMC) cohort (Neuroblastoma public— Versteeg -88, https://hgserver1.amc.nl). The correlation between MYCN and CD71 was evaluated in different stages and the expression graphs were plotted using GraphPad Prism 5 software (GraphPad Software). The Kaplan curves for survival were drawn for the MYCN and CD71 expressed neuroblastoma samples. Moreover, expression of CD71 was analyzed in the MYCN amplified neuroblastoma tumor samples and plotted by using GraphPad Prism5 software. The expression data of neuroblastoma cell lines were obtained from CCLE dataset (The Cancer Cell Line Encyclopedia by Broad Institute, and the Novartis Institutes for Biomedical Research and its Genomics Institute of the Novartis Research Foundation, http:// www.broadinstitute.org).

Cell culture

Human IMR-32, SH-SY5Y, SK-N-MC, and SK-N-SH neuroblastoma cell lines, human embryonic kidney cells (HEK293) and human embryonic fibroblast cells (HEF) were purchased from Korean Cell Line Bank (KCLB) (Seoul, Korea). SK-N-DZ neuroblastoma cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All the cells were grown at 37°C in a humidified 5% CO_2 incubator. IMR-32 cells were cultured in RPMI-1640 supplemented with 10% FBS and without antibiotics; all the other cells were cultured in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. The cell lines used here were periodically checked for mycoplasma infection using Universal Mycoplasma Detection Kit (ATCC® 30-1012KTM) (ATCC) and we found no cells that were infected.

Transfection

Neuroblastoma cell transfections were with Lipofectamine-3000 (Invitrogen). These included siRNA for CD71, HDAC2 or MYCN, control siRNA, an activated-Rheb (S16H) construct in pCAGIG vector, empty pCAGIG vector, and CD71-overexpression vector and miR183 inhibitor, miR183 mimic and miR-control vectors were transfected into cells depending on the experiment. The transfection protocol was according to the manufacturer's protocol. Briefly, the cells were seeded into 6-well or 96-well cell culture plates in growth medium without antibiotics. Lipofectamine-3000 was diluted in either DMEM or RPMI medium without serum and mixed with siRNA oligo or the vector construct in Lipofectamine-3000 P3000 reagent. The transfection complex was then mixed with media containing the cells. After 6 h, the cells were supplemented with the fresh complete media and incubated for an additional 48 h before various assays.

Cell viability, proliferation assay

Cells were seeded into 96-well flat bottom microtiter plates with 1×10^4 cells per well and were then treated with various agents. At the end of each incubation, thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich) was added to each well at a concentration of 1 µg/ml; the plate was then incubated for 3 h at 37°C in the dark. The reaction color was then developed using DMSO and the optical density (OD) was measured at 570 nm with a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

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Cell morphology analysis

Cells were seeded at 1×10^5 cells per well into 24-well plates at 37°C in a humidified 5% CO₂ incubator and treated with various reagents. Cell morphology was then examined under a bright-field inverted microscope (Model IX70; Olympus, Tokyo, Japan) and digitally photographed.

Mitochondria fusion study

SK-N-MC cells were seeded into a 24-well plate at 1×10^4 cells per well and were treated with GA for 6 h. The mitochondria were then stained with MitoTracker Red stain (M22425; Thermo Fisher) and were photographed under an inverted fluorescence microscope (Model IX70; Olympus) at 40X.

Mitochondrial membrane potential analysis

SK-N-MC and SH-SY5Y cells (1×10^6 cells for each well) were seeded into 6-well dishes. After treatment of the cells with various reagents, they were stained with 10 μ M of rhodamine 123 (Sigma-Aldrich) for 30 min in dark. The fluorescence intensity of the cells was measured by flow cytometry (BD FACScalibur, BD Biosciences, San Jose, CA, USA) using the FL-1H channel. The flow cytometry data was then analyzed with Cyflogic software (CyFlo Ltd., Turku, Finland).

Immunocytochemistry

Cells were plated at 1×10^5 cells per well, grown on round coverslips in 24-well dishes. After treatment with different reagents, the cells were then washed with ice-cold phosphate buffered saline (PBS). They were next fixed with 4% paraformaldehyde (PFA) solution for 15 min at room temperature and washed twice with PBS. The cells were next permeabilized with PBS containing 0.25% Triton X-100 for 10 min and washed three times for 5 minutes each with PBS and incubated in 1% bovine serum albumin (BSA) in PBST for 30 minutes to block nonspecific antibody binding. The cells were then incubated overnight at 4°C with the diluted antibody (1:400) (anti-CD71 or anti-EGFR and Phalloidin Red) in 1% BSA in PBST. The cells were then washed three times for 5 min with PBS and incubated in secondary antibody (1:1000) with DAPI (0.1 µg/ml) in 1% BSA for 1 h at room temperature in dark. After washing three times for 5 minutes each in PBS, the coverslip was mounted on a slide with DPX Mounting Medium (Dako North America, Carpinteria, CA, USA) and dried at room temperature. The images of the cells were captured using an Olympus IX70 microscope (Olympus).

Protein extraction and Western blot analysis

Cells were seeded into 6-well plates at 1×10⁶ cells per well. The treated cells were lysed on ice with radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl (pH 8) that included a cocktail of protease inhibitors (Roche, Basel, Switzerland). The cell lysates were then clarified by centrifugation at 4°C for 20 min at 13000×g with the supernatants collected. The protein concentration of the lysates was measured by the Bradford assay (Bio-Rad, Richmond CA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8-15% reducing gels) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking the membrane at 5% non-fat milk (Difco / Becton Dickinson, Franklin Lakes, NJ, USA), it was incubated with the primary antibody overnight at 4°C and washed with TBST (10 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.6). The membrane was then incubated with the appropriate secondary antibody at room temperature for 3 h and washed again with TBST. The Western blots bands were visualized by enhanced chemiluminescence (ECL) (Luminata Forte; Millipore) and exposed to X-ray film (Fujifilm, Tokyo, Japan).

Caspase-3 activity assay

Cells (1×10^5) were seeded into 6-well dishes and allowed to attach to the plate bottom. The cells were then incubated with various agents. Following this incubation, the cells were harvested and permeabilized/fixed in 1 ml methanol at -20°C for 5-10 min. The anti-cleaved caspase-3 antibody was then added at 0.1-10 µg/ml in 3% BSA in PBST for 30 min at room temperature. After this, cells were further incubated with FITC-tagged secondary antibody for 20 min in dark. Fluorescence was measured by flow cytometry using FL-IH filters. Data were analyzed with Cyflogic software (CyFlo).

Immunoprecipitation

Cells were lysed on ice for 30 min in a Nonidet P40 (NP40) buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM NaF, 1 mM Na₃VO₄, 1% NP40, 0.02% NaN₃) containing a protease inhibitor cocktail (Roche). The cell lysates were then centrifuged at 13000×g for 15 min at 4°C and the supernatants were collected. To preclear, the lysates, 1.5 mg of cell lysates were incubated with 100 μ l of protein A-agarose (Santa Cruz Biotechnology) for 3 h at 4°C. Lysates were then further incubated with rabbit anti-ubiquitin antibody (1 μ g antibody/ 1 mg cellular lysate) (Enzo Life Sciences, Farmingdale, NY, USA) for overnight at 4°C. Lysates were again incubated with 20 μ l of protein A-agarose for 1.5 h at 4°C. As a negative control, equivalent amounts of cellular lysates were incubated overnight without antibodies followed by bead incubation. The incubated beads were collected by centrifugation at 200×g for 2 min at 4°C and were washed three times with the NP40 buffer. Samples were eluted from the beads by addition of 40 μ l 1X SDS sample buffer, boiled and were separated on 12% SDS-PAGE gels for Western blot analysis to detect EGFR expression.

Colony formation on low attachment plates

To determine any anti-proliferative effect of GA, the SK-N-MC cells were exposed to GA for 6 h and washed with PBS, harvested and reseeded for colony formation at 100 cells per well in low attachment plates (SPL Life Sciences, Pocheon, Korea) and cultured for the next 7 days. After the incubation, the colonies were observed were then photographed following crystal violet staining/fixation with 4% PFA in PBS.

RNA extraction and quantitative real-time PCR

After transfection, total mRNA was extracted from various neuroblastoma cell lines (IMR-32, SH-SY5Y, SK-N-DZ, SK-N-SH, and SK-N-MC). The cDNA was then made using the miScript II RT Kit (Qiagen) and according to the manufacturer's protocol. Levels of the target message RNA were detected and quantified with SYBR Green RT-PCR kit (Qiagen) containing a universal reverse primer. The expression of message RNA was determined relative to the GAPDH message by the formula: $2^{\Delta Ct} = 2^{-[(Ct of the target gene)-(Ct of GAPDH)]}$, where Ct is the PCR threshold cycle and $2^{\Delta Ct}$ is the relative level for the target gene.

mRNA microarray analysis

IMR-32 cells were untreated or treated with 1.4 μ M GA and 2.4 μ M vorinostat for 4 h. Total RNA from 10⁶ cells in triplicate in 6-well plates of IMR-32 cells was extracted with 0.5 ml of RNAlater (Gibco) as part of the extraction kit (miRNeasy mini kit) (Qiagen). Extracted RNA was sent to Macrogen Microarray Service (Macrogen, Seoul, Korea) for Affymetrix Human Gene 2.0 ST Array profiling (Affymetrix, Thermo Fisher). Data were collected and normalized using Affymetrix Power Tools (APT) (Affymetrix, Thermo Fisher) and the multi-average (RMA) method. Differentially expressed gene (DEG) analysis was also performed by Macrogen. Expression data was determined using fold changes and for DEG sets, hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity.

Combination index calculation for combination drug-treatment

Combination indices (CI) of vorinostat with GA and panobinostat with GA were calculated by using the Chou-Talalay method with the help of CompuSyn software (CompuSyn, Paramus, NJ, USA). The resulting combination index (CI) theorem of Chou-Talalay described the quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations [37].

Plots and statistical analysis

Results were expressed as mean \pm standard error (SE), calculated with Microsoft Excel (Microsoft, Redmond, WA, USA). Unless noted, all plots were made with Microsoft Excel. Statistical significance was assessed by one-way analysis of variance, followed by post-hoc tests using GraphPad Prism 5 (GraphPad Software). Values of p < 0.01 were considered significant.

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Results

Overexpression of MYCN and CD71 in neuroblastoma cells

Amplification of the Myc family member, MYCN, is found in about 25% of neuroblastoma diagnoses and it correlates with high-risk disease and poor prognosis as it remains the bestcharacterized genetic marker of risk in neuroblastoma [27, 38]. Besides MYCN, another oncogenic factor, CD71 (transferrin receptor 1, TFR1) is overexpressed in neuroblastoma patients (Fig. 1). CD71 and MYCN expression studies using two publically available neuroblastoma cohorts in the R2 database the Academic Medical Center (AMC)- Versteeg cohort (n=88) and a large neuroblastoma cases Kocak cohort (n=649) from the German Neuroblastoma Trial revealed a strong correlation with neuroblastoma aggressiveness (Fig. 1A-B). With Versteeg cohort when separated by the stages, we found a significant correlation with stage 4 (n=40) but not with the other stages (Stage 1, 2, 3 and 4s) (data not shown). While the Kocak cohort was separated by stage, a strong, significant correlation was only found in the International Neuroblastoma Staging System (INSS) stage 3 and 4 patients (Fig. 1C-F). Looking at the expression pattern of CD71, we found the mean expression of CD71 to be higher in the aggressive, later stages of neuroblastoma than with those of the earlier stages (Supplementary Fig. 1A – for all supplemental material see www.cellphysiolbiochem.com). Moreover, 45% of MYCN non-amplified neuroblastoma patient were also overexpressed with CD71 in Versteeg cohort (Supplementary Fig. 1B). Accordingly, the expression of both genes, CD71 and MYCN, was correlated with poor survival of neuroblastoma patients in both cohorts (Fig. 1G-H). Therefore, the rationale for this study was to test a combination of treatments targeting MYCN and CD71 and to assess whether the treatments synergize in reducing cell viability with proliferation in model neuroblastoma cells.

For neuroblastoma cell lines, CCLE dataset analysis reveals that a particular set of cell lines also co-overexpress MYCN and CD71 (Supplementary Fig. 1B). The relative expressions of CD71 and MYCN were assessed by qPCR in IMR-32, SK-N-DZ, SH-SY5Y, SK-N-MC, and SK-N-SH cell lines (Supplementary Fig. 1C). The IMR-32 and SK-N-DZ cells had a relatively high MYCN expression in culture and have been documented carrying multiple copies of the MYCN gene [39, 40]. In contrast, SH-SY5Y NB cells displayed a relatively low basal level of MYCN expression and contain a single copy MYCN [41, 42]. For SK-N-MC and SK-N-SH, there was no detectable expression of MYCN. For all the neuroblastoma cell lines tested, there was detectable CD71 expression, but in certain cell lines, the levels were higher (Supplementary Fig. 1C).

To target CD71 and MYCN, IMR-32 cells were either untreated or were separately treated for 4 h with IC50 values of gambogic acid and vorinostat. Microarray analysis was performed to identify the enriched pathways for each treatment (Supplementary Fig. 2A-C). The KEGG pathway analysis identified differential and non-overlapping expression patterns for each treatment for pathways significant for cellular growth/proliferation and death (Supplementary Fig. 2B).

Compared with non-cancer cells, gambogic acid preferentially causes a reduction in cell proliferation/viability in neuroblastoma cells

For targeting CD71 overexpressing cells, gambogic acid (GA) a ligand for CD71 was selected; GA is known to have anti-proliferative properties in various tumor types [24-27] and we seek to exploit the link for overexpression of CD71 and the growth inhibiting properties of GA in neuroblastoma cells. Interestingly, while testing different doses of GA, we found that the sensitivity to GA was higher in MYCN low-expresser cells than for MYCN highexpresser ones according to the MTT assay (Fig. 2A and Table 2). This was also reflected in the colony-forming ability of SK-N-MC cells being significantly reduced with GA treatment (Supplementary Fig. 3A). As many anti-cancer agents also exhibit substantial toxicity towards normal cells, we performed a dose-dependent cytotoxicity assay with GA on human embryonic kidney (HEK-293) cells and human embryonic fibroblast (HEF) cells as models for non-tumor cells. As indicated in Fig. 2A, GA also reduced the viability with proliferation



Fig. 2. Gambogic acid preferably targets neuroblastoma cells through CD71. (A) Neuroblastoma cells (IMR-32, SK-N-DZ, SH-SY5Y, SK-N-SH, and SK-N-MC), **HEK293** and HEF cells were treated with different (0 to 3.2μ M) doses of GA and the relative percentage of viability with proliferation was calculated by following the formula: % viability, proliferation = (optical density (OD) of the drugtreated sample/OD of the control sample) × 100. The values were plotted with a log dose and the curve-fit was by GraphPad Prism 5. (B) SK-N-MC cells were treated with 0.5 μ M of



GA, and incubated for different time intervals and fixed. Expression of CD71 was observed using the anti-CD71 antibody. DAPI was used to stain the nuclei. Photographs were obtained by a confocal microscope at 40X magnification. (C) GA-treated SK-N-MC cells were stained with Mito-Tracker Red and imaged under an inverted fluorescence microscope at 40X. (D) GA sensitive SK-N-SH cells were transfected with CD71 overexpression vector or siRNA against CD71 or control vector and with 0.5 μ M of GA. Expression of CD71 was confirmed in the transfected cells by Western blot and the relative percentage of viability with proliferation was calculated by MTT assay. The results were expressed as mean ± S.E. (E) MYCN overexpressed IMR-32 cells were transfected with siRNA against MYCN or control siRNA vector with or without GA and Western blot was performed to determine the expression of MYCN; the relative percentage of viability with proliferation was calculated by MTT assay. The results were expressed as mean ± S.E.

of HEK293 and HEF cells in culture, but at much higher doses than those required for inhibiting NB cells and the LD50 dose was not reached for these cells (Table 2). The doses of GA required for 50% inhibition of tumor cell combined viability with proliferation was from 0.7–1.5 μ M, depending on the cell type tested (Fig. 2A and Table 2). This data suggested that GA was selective towards reducing viability

Table 2. LD50 dose of GA; treatment time, 6 h

Sl. No.	NB cell lines	R ² value	LD50 dose (µM)	MYCN status
1.	SK-N-MC	0.9913	0.79	Low expresser
2.	SK-N-SH	0.9876	0.81	Low expresser
3.	SH-SY5Y	0.9856	1.02	Single copy
4.	IMR-32	0.9941	1.36	Amplified
5.	SK-N-DZ	0.9937	1.58	Amplified

with proliferation for several neuroblastoma tumor cell lines. We also sought to define the pathway(s) responsible for the effect of GA on these cells and demonstrate the relationship between GA potency to the expression of CD71 in these cells.

CD71 targeting by gambogic acid leads to changes in CD71 expression

To explore the mechanism of GA treatment affecting the treated cells, it was hypothesized that GA first binds to CD71 and then reduces the expression of CD71, possibly restricting the

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supply of transferrin-iron to the cells. To address this, SK-N-MC cells were treated with 0.5 μ M of GA for different time periods and checked for changes in the expression of CD71 by confocal microscopy and FACS analysis. The expression of CD71 in the cells was diminished maximally at 4 h post treatment with GA (Fig. 2B and Supplementary Fig. 3B). As changes in CD71 function may be reflected in mitochondrial morphology [45], GA-treated cells were analyzed with Mito-tracker Red to assess the fusion state of their mitochondria. It was noted that mitochondria aggregation in the treated cells was significantly reduced (Fig. 2C). Moreover, the mitochondrial membrane was significantly depolarized in the GA-treated cells compared with control cells, and there was a 36.6% decrease in the mitochondrial membrane potential (Supplementary Fig. 3C).

To check for changes in iron homeostasis upon GA binding, the cells were placed under modified iron levels in their growth media. For this, SK-N-MC cells were pretreated with either the iron chelator DFO or with iron(III) sulfate for 24 h and then with GA for 6 h. When iron was chelated from the media, depolarization in the mitochondrial membrane with GA was higher and it reached 57.9% of control (Supplementary Fig. 3C). When there was excess iron present in the growth media as with iron(III) sulfate, GA lost some of its potency to depolarize the mitochondrial membrane and the membrane depolarization was only 18.1% of control (Supplementary Fig. 3C). This trend was also seen with changes in cell viability, proliferation and morphology of the treated cells (Supplementary Fig. 3D-E). Therefore, there was a possibility of competition for binding to CD71 between the loading-iron complex and GA, although we did not explore this further.

CD71-dependent cell death by gambogic acid in neuroblastoma cells

To confirm changes in levels of CD71 on sensitivity to GA, GA-sensitive SK-N-SH cells were transfected either with siRNA against CD71 or an overexpression vector for CD71 (Fig. 2D). Cells targeted with siRNA against CD71 had significantly reduced sensitivity to GA and, in contrast, CD71 overexpression significantly increased sensitivity to GA in SK-N-SH cells. This implied that expression level of CD71 was, for the most part, contributing to the sensitivity of cells towards GA, with CD71 being the major surface receptor of GA for entry to these cells [26].

We also observed MYCN overexpressed or amplified cell lines being relatively resistant towards GA treatment; therefore, we set out to determine if changing the endogenous levels of MYCN affects GA mediated cell death. MYCN was first silenced by siRNA in IMR-32 cells which had endogenous overexpressed or amplified MYCN levels; those cells were then treated with GA. Combined viability with proliferation of the IMR-32 cells was significantly reduced in MYCN silenced cells compared with control siRNA treated cells when treated with GA (Fig. 2E). This implied that sensitivity to GA significantly increases if MYCN is present at relatively low levels and when also when CD71 is abundantly expressed or overexpressed.

ER stress-mediated loss of cell viability/proliferation with GA is JNK1/2 dependent

As part of exploring the mechanism of cell death by GA, the possibility of ER stress induction by GA was considered and studied. In the presence of GA (0.5 μ M), an increase in ER stress markers (CHOP, IRE1 α , and ERO-L α) was seen (Fig. 3A). A comparison with the conventional ER stress-inducing agent tunicamycin (Tm) was also made. There were similar changes in the above markers with GA and with Tm treatment (Fig. 3A). To assess the contribution of ER-mediated stress on cell viability/proliferation changes with GA, we first treated SH-SY5Y and SK-N-MC cells with an ER stress inhibitor, salubrinal (Sal). In salubrinal pretreated cells, GA mediated cytotoxicity was abolished in both SH-SY5Y and SK-N-MC cells (Fig. 3B). This indicated that GA-mediated induction of ER stress was the major contributor to cell death due to GA.

Lowering the CD71 expression has also been shown to activate JNK [43, 44]. To determine the contribution of JNK1/2 activation to GA-mediated reduction in cell viability and proliferation, we pretreated cells with the JNK1/2 inhibitor SP600125 and performed a viability/proliferation assay on GA-treated cells (Fig. 3C). Viability/proliferation assay and



Fig. 3. Gambogic acid activates JNK and ER stress to induce cell death. (A) SH-SY5Y SK-N-MC and cells were treated with ER stress inducer Tm for 24 h or with GA for 6 h and expressions of ER stress-related proteins (CHOP, ERO, and IRE) were evaluated using Western blot. (B) SH-SY5Y and SK-N-MC cells were pretreated with stress inhibitor, ER Sal, for 24 h and then with GA for 6 h. Cell viability/proliferation assav was performed the relative and percentages of viability with proliferation were calculated and plotted as a histogram using GraphPad Prism 5. (C-E) Neuroblastoma cells were treated with INK inhibitor-SP600125 incubated for 24 h



and GA-treated cells were incubated for 6h. In the combination treatment sets, cells were first exposed to SP600125 for 24 h and then GA was added for 6 h. (C) Cell viability/proliferation assay was performed and the relative percentages of viability with proliferation were calculated and plotted as a histogram by using GraphPad Prism 5. (D) Cellular morphology was imaged at 40X after the treatment period. Yellow arrows are indicative of the apoptotic cells. (E) Expression of different ER stress and apoptosis-related proteins were examined with Western blot. Actin was used as a loading control. (F) Cells were pretreated with pancaspase inhibitor Z-DEVD-FMK and then with GA to find the involvement of caspase dependence in the GA-death pathway. Cell viability/proliferation assay was performed and the relative percentages of viability with proliferation assay was performed and the relative percentages of viability with proliferation assay was performed and the relative percentages of viability with proliferation assay was performed and the relative percentages of viability with proliferation were calculated and plotted as a histogram by using GraphPad Prism 5.

microscopic analysis revealed that the reduction of JNK1/2 phosphorylation by SP600125 significantly attenuated cell death and morphology changes (rounding-up) caused by GA in both SH-SY5Y and SK-N-MC cells (Fig. 3C-D). Later, we examined activity changes for JNK1/2 following exposure to GA. As seen in Fig. 3E, phosphorylation of JNK1/2 was increased by GA treatment along with the increased expression of ER stress markers CHOP, IRE1 α and ERO-L α (Fig. 3E). With cells pretreated with JNK1/2 inhibitor SP600125, GA was unable to increase expression of CHOP and IRE1 α , except for ERO-L α (Fig. 3E). A pan-caspase inhibitor, Z-DEVD-FMK, also suppressed cell death from GA (Fig. 3F), implying that GA depended on caspase pathways to induce cell death.

GA influence on the target cells is only partially dependent on the mTORC1 activity

To see if the activity of mTOR kinase was also altered by GA treatment, we monitored changes in levels of phosphor-S2448 and S2481 of mTOR kinase and phosphorylation of S6K, indicators of mTOR kinase activity. Treatment with GA increased phosphorylation of



Fig. 4. Gambogic aciddeath pathway was partially controlled by the activity of mTOR kinase. (A) SH-SY5Y and SK-N-MC cells were treated with GA for 6 h and phosphorylation of mTORC1 and its downstream protein p-S6K were examined by Western blot. (B-F) SH-SY5Y and SK-N-MC cells were either treated with rapamycin or transfected with Rheb (S16H) and then with GA for 6 h. (B-C) expression of p-JNK and ER stress marker CHOP was evaluated by Western blot. JNK and Actin were used as a loading control. (D) Cell viability/ proliferation assav was performed and the relative percentages of viability with proliferation were calculated and plotted as a histogram by using GraphPad Prism



5 software. (E) Caspase-3 activity was determined by indirect staining protocol using a flow cytometer and the data were plotted as a histogram using GraphPad Prism 5 software. (F) Cell morphology of SH-SY5Y and SK-N-MC was imaged to check the apoptotic structure at 40X magnification. Yellow arrows are indicative of the apoptotic cells.

mTOR and S6K in both SH-SY5Y and SK-N-MC cells (Fig. 4A). To understand the role of mTOR kinase in JNK1/2 and ER stress signaling, SK-N-MC cells were either pretreated with mTOR inhibitor rapamycin or transfected with mTOR activator Rheb S16H vector, then treated with GA. We found that GA's potential to phosphorylate JNK1/2 was not diminished when mTOR activity was modulated by rapamycin or Rheb S16H. For the ER stress marker, CHOP, a similar effect was also observed, implying that mTOR's involvement, if any, on GA-cell death pathway, was downstream of JNK1/2 and ER stress activation (Fig. 4B-C). Increases in ER stress due to GA treatment of the cells did not depend on mTORC1 activity changes, shown by directly activating the mTORC1 complex and increasing its associated kinase activity. To assess the involvement of mTOR signaling in GA-triggered cell viability with proliferation read changes, we pretreated cells with mTORC1 inhibitor rapamycin before GA treatment and looked for an effect on viability/proliferation changes due to GA. Rapamycin treatment blunted some of the viability/proliferation loss due to GA (Fig. 4D), and the activity levels of caspase 3 were also lower in rapamycin pretreated cells in presence of GA (Fig. 4E) for both SH-SY5Y and SK-N-MC cells. Partial resistance to rapamycin was also seen with the pattern of morphology changes for cells with various treatments (Fig. 4F). Conversely, viability with

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Fig. 5. Gambogic acid treatment induces ubiquitination of the EGF receptor to degrade it. (A) Expression of EGFR was evaluated in IMR-32, SH-SY5Y and SK-N-MC cells after GA administration by Western blot. Actin was used as a loading control. After GA administration to IMR-32. SK-N-MC, and SH-SY5Y neuroblastoma cells. total ubiquitin protein was immunoprecipitated from their cell lysates. EGFR expression was determined by Western blot. (B) SH-SY5Y cells were treated with 0.5 µM of GA and incubated for 6 h and fixed. Expression of EGFR was observed using an anti-EGFR antibody.



DAPI and Phalloidin Red were used to stain the nuclei and actin filaments, respectively. EGFR expression in the plasma membrane of the cell (white arrows) before GA treatment; post 0.5 μ M GA treatment at 6 h, EGFR expression is localized in the cytosolic and perinuclear compartments (white-outline arrows). Photographs were obtained by a confocal microscope at 63X magnification. (C) Phosphorylation of EGFR at Ser1046/1047 and Tyr1068 was determined in IMR-32, SH-SY5Y and SK-N-MC cells by Western blot with GA. Actin level was used as the loading control.

proliferation read loss due to GA was elevated and caspase-3 activity was increased in the presence of mTOR activator Rheb S16H (Fig. 4D-E) in SH-SY5Y and SK-N-MC neuroblastoma cells. This indicated that basal level increases in mTOR activity could augment the activation of the caspase 3 seen with GA. The changes due to the activation of caspase 3 were more dramatic than the changes in viability with proliferation, although the trends were similar (Fig. 4D-E). Microscopic analysis of the cells also confirmed the changes seen with the MTT assay (Fig. 4F).

Gambogic acid treatment degrades EGFR due to increased phosphorylation of Ser1046/1047 EGFR

To examine the growth inhibiting property of GA, we also looked for any changes in the levels of EGF receptor, EGFR, as EGFR is overexpressed in neuroblastoma samples and GA has been documented to lead to changes in the expression of this receptor [45]. GA administration inhibited EGFR expression as confirmed by Western blotting in the three different NB cell lines tested (Fig. 5A). This reduction was for more than 50% of the total EGFR in the different cell lines tested as detected by Western blotting. EGFR is prone to ubiquitination as part of its degradation pathway [46, 47], and here, we examined the levels of ubiquitinated EGFR by co-immunoprecipitation with an anti-ubiquitin antibody. In the GA-treated neuroblastoma, the extent of ubiquitinated EGFR was significantly higher (Fig. 5A). We also confirmed the increased phosphorylation of EGFR at Ser1046/1047 with GA treatment, as these serine residues have been associated with increased susceptibility of EGFR to degradation (Fig. 5C) [48, 49]. Before the 0.5 μ M GA treatment, EGFR expression is highly localized in the

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plasma membrane of the cell (depicted by white arrows); post GA treatment at 6 h, EGFR expression has mostly vanished from the plasma membrane and is localized in the cytosolic and perinuclear compartments (shown by white-outline arrows) (Fig. 5B). In IMR-32 cells, p-EGFR (Ser1046/1047) expression was increased post-GA administration; this was first detectable at 15 min, peaking at 3 h and remained high at 6 h after treatment (Supplementary Fig. 4). Meanwhile, total EGFR protein expression levels steadily decreased after GA addition, a reflection of increased degradation of EGFR in these cells. The p-EGFR Tyr1068 signal was also diminished by GA (Fig. 5C). This data is interpreted as a treatment by GA leading to ubiquitination-dependent degradation of a significant portion of cellular EGFR in the tested neuroblastoma cells.

Downregulation of MYCN expression by histone deacetylases inhibitor (HDACi), vorinostat, reduces survival of neuroblastoma cells

In various cancer types, MYCN's mechanism of action silences tumor suppressor genes by recruitment of DNA methyltransferases and elevation of HDAC activity [50, 51]. This suggests a possible therapeutic role for HDACi in MYCN overexpressing cells. Here, we used vorinostat (suberoylanildehydoxamic acid/ SAHA), a U.S. Food and Drug Administration (FDA)-approved drug for many cancer types [52, 53], to study the susceptibility of MYCNhigh and MYCN-low expressing NB cells to vorinostat in terms of cell combined viability with proliferation changes. In this analysis, vorinostat could reduce cell viability/proliferation in all the NB cell types tested, and predictably, IMR-32, SK-N-DZ, and SH-SY5Y displayed the most susceptibility compared to SK-N-MC and SK-N-SH cells (Fig. 6A, Table 3). For gauging relative MYCN expression in the tested cells, qPCR was used. The relative expression of MYCN was found to be highest in IMR-32 cells (Supplementary Fig. 1C) and they also showed the highest sensitivity to vorinostat (Table 3). Cells treated with vorinostat for 24 h had reduced expression of both MYCN message in IMR-32 and SK-N-DZ cells as confirmed by qPCR (Fig. 6B). Taken together, MYCN high expresser cells were more sensitive towards vorinostat, possibly reflecting their increased dependence on MYCN pathways for proliferation/ survival. In support of this observation, a transient MYCN siRNA transfection of the test cells significantly diminished the potency of vorinostat in affecting the viability/proliferation of the cells and MYCN overexpression significantly enhanced the potency of vorinostat against neuroblastoma cells (Fig. 6C-D).

We checked the extent of histone H3 acetylation as a means of the specific action of vorinostat on HDAC activity in the target cells. The extent of acetylated histone H3 was assayed by Western blotting, and vorinostat was able to increase acetyl-histone H3 levels with the doses used (Fig. 6E). As it is known that MYCN activates the PI3K-Akt pathway to induce cell proliferation and drug resistance in neuroblastoma cells [54–57], we checked the activity/phosphorylation of Akt and PI3K in the vorinostat-treated cells. Vorinostat was able to reduce the phosphorylation of Akt Ser473 and Thr308 residues and also reduced phosphorylation of PI3K (Fig. 6E). Thus, with vorinostat, the anti-apoptotic PI3K-Akt pathway was inhibited with inhibition of MYCN levels, as vorinostat was able to reduce the signals delivered by MYCN in promoting cancer cell survival/proliferation.

Vorinostat treatment involves miR183 as a downstream effector of HDAC2 and MYCN silencing for cell death

Cooperation between HDAC2 and MYCN is important in suppressing the pro-apoptotic pathway of miR183 and it was previously shown that HDACi treatment upregulates miR183 to induce cell death and was elevated after downregulation of HDAC2 [58]. To check for changes in miR183 expression, two different HDACi's (vorinostat and panobinostat) were used against three different neuroblastoma cell lines, where two were MYCN amplified (IMR-32 and SK-N-DZ), and the other one containing a single copy of MYCN (SH-SY5Y). Treatment with vorinostat and panobinostat significantly upregulated miR183 expression in the MYCN amplified cells as seen by qPCR (Fig. 7A). Silencing of HDAC2 by siRNA also led to an increase in miR183 expression in the MYCN amplified cells but not in SH-SY5Y cells (Fig. 7C). To find



Fia. 6. Vorinostat was more effective in MYCN overexpressors. (A) Neuroblastoma cells were treated with different (0 to 16 μ M) doses of vorinostat and the relative percentage of viability with proliferation calculated were bv following the formula: % viability, proliferation = (optical density (OD) of the drug-treated sample/ OD of the control sample) × 100. The values were plotted in log scale and curvefit was obtained by GraphPad Prism 5. (B) RNA expression of MYCN was examined in IMR-32 and SK-N-DZ cells by qPCR



after vorinostat treatment at different concentrations and normalized with GAPDH as a loading control. The histogram was plotted by GraphPad Prism 5. (C) Cellular morphology was examined after vorinostat (2 μ M) treatment of the neuroblastoma cells. Photographs were imaged with an inverted microscope at 40X magnification. The red arrows are indicative of apoptotic cells. (D) IMR-32 cells were transfected with siRNA and an overexpression vector of MYCN (MYCN OX) and then treated with vorinostat (2 μ M) for 24 h. Expression of MYCN was evaluated by Western blot. The cell viability/proliferation assay was performed and the relative percentages of viability with proliferation were calculated and plotted as a histogram with GraphPad Prism 5. (E) Expressions of acetyl-histone H3, p-Akt (Ser473 and Thr308), Akt, p-PI3K, and PI3K were examined by Western blot after administration of different doses of vorinostat for 24 h. Actin was used as the loading control.

a role of miR183 on vorinostat mediated cell death, the cells were transfected with miR183 inhibitor/mimic and were then treated with vorinostat. Overexpression of miR183 by itself reduces the cell viability/ proliferation in the IMR-32 and SK-N-DZ cells and their cell morphology was also changed (Fig. 7B and Fig. 7D-E). In the vorinostat/miR183 mimic co-treated group, the cell viability/proliferation was

Table 3. LD50 dose of vorinostat; treatment time, 24 h

Sl. No.	NB cell lines	R2 value	LD50 dose (µM)	MYCN status
1.	SK-N-MC	0.9233	Not reached	Low expresser
2.	SK-N-SH	0.9420	Not reached	Low expresser
3.	SH-SY5Y	0.9873	4.48	Single copy
4.	IMR-32	0.9689	2.37	Amplified
5.	SK-N-DZ	0.9600	3.76	Amplified

significantly reduced compared with control and miR183 alone treated cells. Alternatively, silencing of miR183 reduced the potency of vorinostat in both IMR-32 and SK-N-DZ cells (Fig. 7D-E). Silencing of miR183 also attenuated cell death caused by HDAC2 silencing in neuroblastoma cells (Fig. 7F).

To understand the role of MYCN in regulating miR183 expression in neuroblastoma cells, we either silenced MYCN by siRNA or overexpressed MYCN by an overexpression vector for MYCN in IMR-32 cells. Treatment with MYCN siRNA upregulated miR183 expression (Fig.

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Fig. 7. Vorinostat increased miR183 expression in MYCN overexpressed cells to reduce cell combined viability with proliferation reads. (A) miR183 expression was examined by qPCR following dosing of HDACi for 24 h. Vorinostat $(2 \mu M)$ and panobinostat $(2 \mu M)$ were able to increase miR183 expression in IMR-32 and SKcells N-DZ but not in SH-SY5Y cells. (B) Different neuroblastoma cells were either transfected with miR183 inhibitor oligoes or with miR183 mimic. Expression of miR183 was examined by qPCR GAPDH was and used to normalize the signal. (C)miR183 expression was examined by qPCR in IMR-32, SK-N-DZ, and SH-



SY5Y cells after transfecting them with HDAC2 siRNA. GAPDH was the housekeeping control. (D) IMR-32 cell morphology was examined after treatment with vorinostat (2 μ M), miR183 mimic and with miR183 and vorinostat together. The photographs were taken by an inverted microscope at 20X magnification. The yellow arrows are indicative of apoptotic cells. (E) IMR-32 and SK-N-DZ cells were treated with vorinostat (2 μ M), miR183 inhibitor oligoes or miR183 mimic alone or together. The cell viability/proliferation assay was performed and the relative percentages of viability with proliferation were calculated and plotted as a histogram with GraphPad Prism 5. (F) IMR-32 and SK-N-DZ cells were either transfected with HDAC2 siRNA or with miR183 inhibitor oligoes or both together for 48 h and then the cell viability/proliferation assay was performed and the relative percentages of viability with proliferation were calculated and plotted as a histogram by GraphPad Prism 5.

8A and C). Alternatively, overexpression of MYCN reduced levels of miR183 in IMR-32 cells (Fig. 8B and C). Silencing of either of MYCN and HDAC2 can elevate miR183 expression (Fig. 8D). Thus, HDACIs reducing cell viability, proliferation of MYCN amplified cells seems to



Fig. 8. MYCN levels modulate negatively miR183 expression IMR-32 cells. in (A-C) IMR-32 cells were transfected with control siRNA/ MYCN siRNA or control vector/MYCN overexpression vector indicated. Cells as were harvested at the times indicated post transfection and were in RNAlater lysed RNA solution. was extracted from the cells and the relative levels of miR183 or MYCN message to that of GAPDH were



then quantified by qPCR according to Materials and Methods. (A-B) miR expression is shown after MYCN silencing (MYCN siRNA) or MYCN overexpression (MYCN OX). (C) Expression of MYCN was downregulated with siRNA transfection and upregulated from the overexpression vector. (D) miR183 expression was examined after co-silencing of HDAC2 and MYCN with their respective siRNAs or control siRNA.

depend on miR183 levels. The transcriptional regulation of MYCN and inhibition of HDAC2 by vorinostat could be linked to the pro-apoptotic miR183 levels.

Pretreatment with vorinostat sensitizes neuroblastoma cells to gambogic acid for increased apoptosis

Targeting multiple pathways in cancer treatment shows promise [59]. Here to target the aggressive MYCN amplified neuroblastoma cells, a combination treatment strategy was adopted. To suppress the MYCN activity, we selected HDACi vorinostat; in the same cells, CD71 was also targeted by GA. We studied the effect of combining GA and vorinostat on viability, proliferation loss on the target cells (treatment protocol described in Fig. 9A). We adopted the Chou-Talalay method for drug combination. The resulting combination index (CI) theorem of Chou-Talalay offers a quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations [37]. After the combination of vorinostat and GA, the combination index (CI) was calculated for cell lines using the CompuSyn software and they were plotted. As shown, the CI was more synergic towards the higher dose of GA than the lowest one (Fig. 9B-C). To see if GA sensitization to HDAC inhibition was specific to vorinostat or to other HDAC inhibitors as well, we tested panobinostat, also an HDACi, in the assays and found that the higher MYCN expressing cells (IMR-32, SK-N-DZ) were also more sensitive to panobinostat (Fig. 9B-C). Panobinostat/GA combination also had a synergistic effect on neuroblastoma cells similar to the vorinostat/GA combination. The combination treatment of HDAC inhibitor and GA also did not show any significant cell death in HEK293 and HEF cells, as models of non-cancer cells (Supplementary Fig. 5A-B). The acetylation of histone H3 with vorinostat and GA treatment was similar to vorinostat alone in both IMR-32 and SK-N-DZ cells (Supplementary Fig. 4C). We found a similar effect with the loss of MYCN expression in IMR-32 cells. Lastly, we examined caspase-3 changes with the combination of vorinostat and GA treatment of the cells and found an increase in the expression of caspase-3 and -9 in IMR-32 cells (Supplementary Fig. 5C). GA treatment also weakly increased the expression of acetyl histone H3 (Supplementary Fig. 5C). The enrichment was significantly

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Fig. 9. Combination vorinostat of or panobinostat and gambogic acid is more effective against the neuroblastoma cells tested. (A) IMR-32 and SK-N-DZ cells both have relatively high levels of MYCN. Vorinostat or panobinostat and GA were added to these cells. (A) Incubation time for vorinostat or panobinostat was 24 h and that for GA was 6 h. For combination treatment. cells were pretreated with vorinostat or panobinostat for 18 h before the GA administration and were then incubated with GA for 6 h more. (B-C) The relative percentages viability with of



proliferation were calculated by the following formula: % viability, proliferation = (optical density (OD) of the drug-treated sample/OD of the control sample) × 100. Values were plotted as a histogram by using GraphPad Prism 5. Combination index (CI) was calculated by using the Chou-Talalay method with CompuSyn software. The calculated CI was calculated and plotted using GraphPad Prism 5.

less than that of vorinostat at the concentrations used. It is not known whether this was due to GA acting as a general HDAC inhibitor. Combining vorinostat with GA proved to be synergistic in MYCN overexpressing neuroblastoma cells in both increased cleaved caspase-3 levels and decreased cell viability, proliferation. Even when combined, the pathways for GA and vorinostat do not seem to intersect in the modulation of CD71 and MYCN levels, respectively, as either agent does not seem to affect the levels of each respective target of the other pathway (Fig. 10).

Discussion

For a high percentage of high risk or aggressive neuroblastoma, MYCN is either overexpressed or its gene is amplified and is thought to be a driver for these cancers [60]. Reducing the expression levels of MYCN could thus be a means of reducing the aggressiveness of these tumors. Another feature of many cancer types including neuroblastoma is surface overexpression of CD71, also known as the transferrin receptor 1, and its increased expression has been correlated with later-stage tumors [8]. To date, only a limited number of studies have defined a role for increases in CD71 levels in neuroblastoma [61, 62]. In addition, such increases in cell surface expression of CD71 could be used as a means of targeted delivery





Fig. 10. Schematic representation of pathway dependency for cell death induction by gambogic acid and vorinostat and their combination treatment. GA works via CD71 dependent JNK pathway for ER stress generation and vorinostat works via HDAC and MYCN pathway to induce apoptosis.

of various cancer therapeutic agents. In this study, singly and in combination, we tested neuroblastoma cells that overexpress CD71 and MYCN for targeted killing by various agents affecting and being dependent for their action on these two targets. Surface CD71 allowed binding and internalization of gambogic acid and delivery to the cells of a cytotoxic signal. Vorinostat treatment, in turn, lowered the expression levels of MYCN in the treated cells, leading to an MYCN-dependent decrease in cell viability, proliferation. When combining GA and vorinostat in treating the cells, vorinostat reduced MYCN transcription and the cells became even more sensitive to GA therapy. The suitability of GA as a therapeutic agent via clinical trials still needs to be demonstrated; however, GA is being considered in cancer therapy as it enters phase II clinical trials in China for lymphatic sarcoma, breast cancer and various skin lesions [63, 64]. In this report, we elucidated the potential of GA as a therapeutic agent for neuroblastoma, particularly when combined with the HDAC inhibitor, vorinostat. As GA is a known ligand for CD71 and CD71 is overexpressed in a significant number of neuroblastoma samples [26], the use of GA may be suitable for targeting such tumor cases.

On the proximal events in the mechanism of GA, from a previous report, EGFR was shown to be a positive regulator of CD71 in non-small cell lung cancer cells [65]. In a separate report, GA-administration also led to the degradation of EGFR protein via an AMPK-dependent upregulation of LRIG1, a regulator of EGFR levels in glioma cells [43]. In our study, GA

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administration led to a reduction in EGFR levels and increased phosphorylation of EGFR at Ser1046/1047 residues. These two residues of EGFR having been associated with increased susceptibility of EGFR to degradation [48]. This observation establishes a possible rationale for the proximal events in GA action in neuroblastoma cells with GA leading to inactivation of EGFR, this in turn bringing a reduction in cellular levels of CD71. A subsequent reduction in cellular iron uptake then impacts proliferation and apoptosis.

Our study also explored the signaling properties of GA with respect to various MAP kinases: we showed that GA activates INK. GA also reduces the phosphorylation of MEK and ERK. However, the reduction in phosphorylation of MEK and ERK from GA treatment did not affect the increases in ER stress or the onset of cell death by GA (data not shown). On the other hand, the phosphorylation increase in INK turned out to be required for the GA-mediated increase in ER stress and induction of cell death. This implies that INK phosphorylation was required in GA-mediated cytotoxicity against neuroblastoma cells. This is on par with documented INK's role in activating various ER stress-related proteins and contributing to cell death [66, 67]. The cell death response via JNK-IRE signaling is via an increase in CHOP transcription [68] with CHOP inducing apoptosis with direct inhibition of Bcl-2 levels [69] and reducing the Bcl-2/Bax ratio. Of note, the stress signals from the ER to the mitochondria can lead to apoptosis in mitochondrial-mediated apoptosis regulated by the Bcl-2 family of proteins [70, 71]. With GA treatment, we also observed activation of mTORC1; however, mTORC1 only played a partial role in GA-mediated death. With mTORC1 inhibitor rapamycin pretreatment, the apoptosis induction by GA was only partially attenuated. Rapamycin did not alter the stress response changes due to GA for ER stress markers ERO, IRE, and CHOP; this could be because the mTORC1 activity may both contribute and hinder the unfolded protein response (UPR) in a complex interaction between mTORC1 and ER stress [72].

We have seen the efficacy of GA being higher when MYCN levels were lower in neuroblastoma cells, implying that MYCN may modulate the susceptibility to GA. By reducing MYCN cellular levels with vorinostat, a synergy was seen in increased cytotoxicity from GA. A similar example is with the administration of panobinostat, an HDACi similar to vorinostat, it reduced the MYCN-related c-Myc expression in melanoma cells [32]. MYCN and HDAC2 together block miR183 transcription and reduce its pro-apoptotic characteristics [58]. MYCN and HDAC2 may also cooperate in suppressing p53 Ser46 phosphorylation [73], although we did not show this. MYCN may also be working via another HDAC family member, HDAC5, in repressing CD9 transcription that results in triggering of invasion and metastasis in neuroblastoma [74]. Therefore, MYCN acts as a transcriptional regulator for various pro-apoptotic factors that make the cancer cell more aggressive and resistant to therapy. Vorinostat by repressing MYCN expression, predominantly in MYCN overexpressed or amplified neuroblastoma cells, may act as an effective means of countering the anti-apoptotic effects of MYCN in the target cells.

The treatment of cells with HDAC inhibitor vorinostat followed by GA caused a synergistic cell growth inhibition for MYCN high expressers. To see if the sensitization from HDAC inhibition was specific only to vorinostat or it worked with other HDACi's as well, we tested panobinostat and similarly found that the higher MYCN expressing cells (IMR-32, SK-N-DZ) were more sensitive to panobinostat. HDACi's have been shown to induce differentiation, cell-cycle arrest, and apoptosis and to inhibit migration, invasion, and angiogenesis in multiple cancer cells studied [75]. HDACi's alone and in combination with a variety of anticancer drugs are now being tested in multiple clinical trials for hematological and solid tumors. Vorinostat was the first HDAC inhibitor approved by the US FDA for treating cutaneous T-cell lymphoma (CTCL) and is now being tested for other malignancies as well [52].

Pathways that drive cancer progression and relapse in high stage neuroblastoma in pediatric groups may depend on pathways with transcriptional misregulation in bulk cancer or cancer stem cells [76]. GA and vorinostat may have the ability to regulate these pathways for cancer relapse in high stage neuroblastoma (Supplementary Fig. 2C). Although only tested in cell lines, the lower cytotoxicity of these drugs against normal cells also indicates a safety margin for these drugs; however, a preclinical study for safety for a combination

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therapy needs to be completed. This includes a study to test the proposed combination of drugs for this class of cancers in an *in vivo* setting. Our results present a novel means of targeting neuroblastoma cells for cell death with GA and the HDAC inhibitor, vorinostat. We provide a data-driven rationale in the observed cytotoxicity seen with these agents and propose preclinical testing of combination therapy with vorinostat and GA in MYCN amplified neuroblastoma.

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Author Contributions: K.B. carried out most of the cellular, molecular and biochemical experiments and analyzed the data; K.H. carried out a part of the immunocytochemistry experiments; A.S. wrote the manuscript and performed part of the cellular experiments, and S.-O. H. designed the project and led the team to accomplish it. All authors reviewed the manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

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