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# Identification of new therapeutic targets and natural compounds against diffuse intrinsic pontine glioma (DIPG)

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ABSTRACT

Diffuse Intrinsic Pontine Glioma (DIPG) is a highly aggressive pediatric brainstem tumor which accounts for about 10–20% of childhood brain tumors. The survival rate for DIPG remains very poor, with a median survival of less than 1 year. The dismal prognosis associated with DIPG has been exacerbated by the failure of a large number of clinical trials to meaningfully improve survival compared with radiotherapy, the current standard of care for DIPG. In the current study, we screened a natural product library and for the first time identified 6 natural compounds displaying inhibitory effects on DIPG proliferation and anchorage-independent growth through inducing tumor cell apoptosis and cell cycle arrest. Subsequent RNA-Sequencing and functional validation revealed the molecular mechanisms of these compounds with anti-DIPG activities, and identified new cellular factors such as Fibronectin 1 (FN1) and Eukaryotic translation initiation factor 3 subunit C-like (EIF3CL), required for DIPG survival as potential therapeutic targets. Our study provides promising directions to fight against this deadly pediatric cancer.

## 1. Introduction

Diffuse intrinsic pontine glioma (DIPG) represents a highly aggressive pediatric brainstem tumor which accounts for about 10–20% of all childhood brain tumors [1–3], mostly seen in children at 5–10 years old. The survival rate for DIPG remains very low, with a median survival of less than 1 year. The current standard of treatment, radiotherapy, unfortunately cannot improve the prognosis of DIPG. Moreover, the treatment of DIPG with chemotherapy has failed to show benefits beyond radiation therapy over the past decades [4]. In addition, we do not yet fully understand the mechanisms for DIPG initiation and progression, especially those cellular factors essential for the survival of these pediatric brain tumor cells. Therefore, the development of new therapeutic approaches, including identification of new targets, is of great importance for improving DIPG treatment. However, due to the sensitive location of DIPG, the lack of surgical specimens, clinical databases or patient-derived cell lines, our understanding of the biology of these brain tumors and the development of targeted therapies has been greatly hindered.

Increasing evidence has supported that many natural products from plants or other resources display anticancer activities, or enhance the efficacy of chemotherapy as well as other treatments [5–7]. In contrast, to our knowledge there is no natural compounds that have been reported with anti-DIPG activities. In the current study, we first screened a natural product library and identified new hit compounds with excellent anti-DIPG activities, including the inhibition of tumor cell proliferation and anchorage-independent growth through inducing apoptosis and cell cycle arrest. The data from RNA-Sequencing analyses revealed the underlying mechanisms of these natural compounds and identified new cellular factors required for DIPG cell survival with further functional validation. Our results provide new insights into the mechanisms of DIPG pathogenesis and promising therapeutic directions for this deadly form of pediatric brain cancer.

## 2. Materials and methods

### 2.1. Cell culture and reagents

DIPG cell lines SF8628 and SF7761 which harbor the histone H3.3 Lys 27-to-methionine (K27M) mutation were purchased from Millipore-Sigma and cultured as recommended by the manufacturer. All experiments were carried out using cells harvested at low (< 20) passages. A compound library consisting of 756 natural products was purchased from Selleck Chemicals, Houston, Texas, USA.

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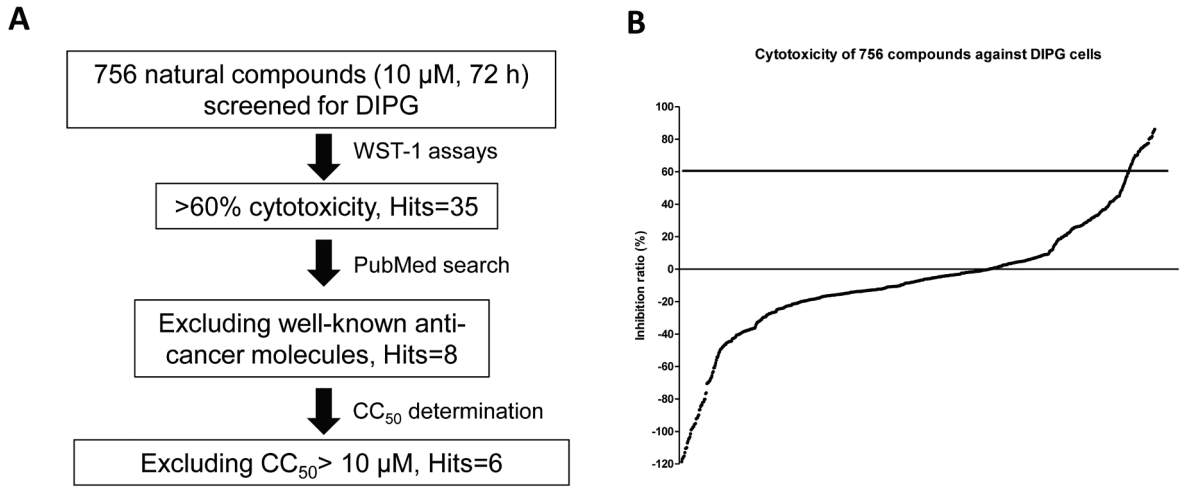


**Fig. 1.** High-throughput screening and identification of new natural compounds with anti-DIPG activities. (A) Diagrams of high-throughput drug screening. The natural compounds in source plates were delivered at 10 μM (final concentration) to 96-well plates seeded with DIPG cell line SF8628 for 72 h treatment, then the cell proliferation status was examined using the WST-1 cell proliferation assays (Roche). (B) Primary screening results of 756 natural compounds against DIPG, which were arranged in order of inhibition rate.

**Table 1**
The final 6 hit natural compounds with prominent anti-DIPG activities.

| Compounds | CAS | $CC_{50}$ (μM)[a] | | Putative mechanisms or targets |
|---|---|---|---|---|
| | | SF8628 | SF7761 | |
| Brefeldin A | 20350-15-6 | 0.028 | 0.052 | A lactone antibiotic, ATPase inhibitor, and improve homology-directed repair. |
| Chaetocin | 28097-03-2 | 0.12 | 0.0004 | A histone methyltransferase inhibitor. |
| Combretastatin A4 | 117048-59-6 | 0.025 | 0.005 | A microtubule-targeting agent that binds to β-tubulin. |
| Gracillin | 19083-00-2 | 3.8 | 0.4 | Unknown, antitumor activity. |
| Protodioscin | 55056-80-9 | 3.3 | 96.3 | A testosterone inducer through interaction with testosterone receptors. |
| Tubercidin | 69-33-0 | 0.43 | 0.014 | An adenosine analogue that inhibits polymerases, with antifungal and antiviral activities. |

[a] $CC_{50}$ represents the 50% cytotoxic concentration determined by using the WST-1 assay.

### 2.2. High-throughput screening

DIPG cell line SF8628 was seeded into 96-well plates for 24 h. Then, the compounds were added into the wells at a final concentration of 10 μM for additional 72-h incubation. The cytotoxicity against DIPG was measured using the WST-1 cell proliferation assays (Roche) according to the manufacturer's instructions. Data was normalized as the inhibition ratio to the DMSO controls.

### 2.3. Cell apoptosis and cell cycle assays

Flow cytometry was used for the quantitative assessment of apoptosis with the FITC-Annexin V/propidium iodide (PI) Apoptosis Detection Kit I (BD Pharmingen) analyzed on a FACS Calibur 4-color flow cytometer (BD Bioscience). For cell cycle analysis, DIPG pellets were fixed in 70% ethanol, and incubated at 4 ˚C overnight. Cell pellets were re-suspended in 0.5 mL of 0.05 mg/mL PI plus 0.2 mg/mL RNaseA and incubated at 37 ˚C for 30 min prior to FACS analysis.

### 2.4. Soft agar assays

The tumor cells anchorage-independent growth abilities were assessed using soft agar assays as described previously [8]. First, a base layer containing 0.5% agarose medium and 5% FCS was poured into six-well plates. Then, 2,000 cells were mixed with 0.4% agarose in Earl's minimal essential medium (EMEM) containing 5% FCS to form a single-cell suspension. After being seeded, the plates were incubated for 4–5 weeks. Colonies were stained with 0.005% crystal violet and photographed under a phase-contrast microscope (Leica DFC320).

### 2.5. RNA-Sequencing and enrichment analysis

RNA-Sequencing with a triplicate for each sample was performed using the Genome Analyzer IIx (Illumina) at LSUHSC Translational Genomics Core (TGC) Facility. The completed RNA-Sequencing data has been submitted to NCBI Sequence Read Archive (SRA# PRJNA590259). Raw sequencing reads were analyzed using the RSEM software (version 1.3.0; human GRCh38 genome sequence and annotation) for quantification of human gene expression as previously described [9]. The EBSeq software was utilized to call statistically differentially expressed genes using a false discovery rate (FDR) less than 0.05. Differentially expressed genes between natural compounds- and vehicle-treated DIPG cells were used as input for the GO_enrichment and KEGG pathway analyses. RNA-Sequencing data of DIPG tumor samples and paired normal brain tissues were obtained from the National Institutes of Health (NIH) Genotypes and Phenotypes (dbGaP) database under the accession number (SRA# SRP136329). Transcript quantification of human genes was conducted as previously described [9].

### 2.6. RNA interference (RNAi)

For RNAi assays, *FN1* or *EIF3CL* On-Target plus Smart Pool small interfering RNA (siRNA; Dharmacon) or negative control siRNA at 50 nM were delivered using the DharmaFECT transfection reagent as recommended by the manufacturer.

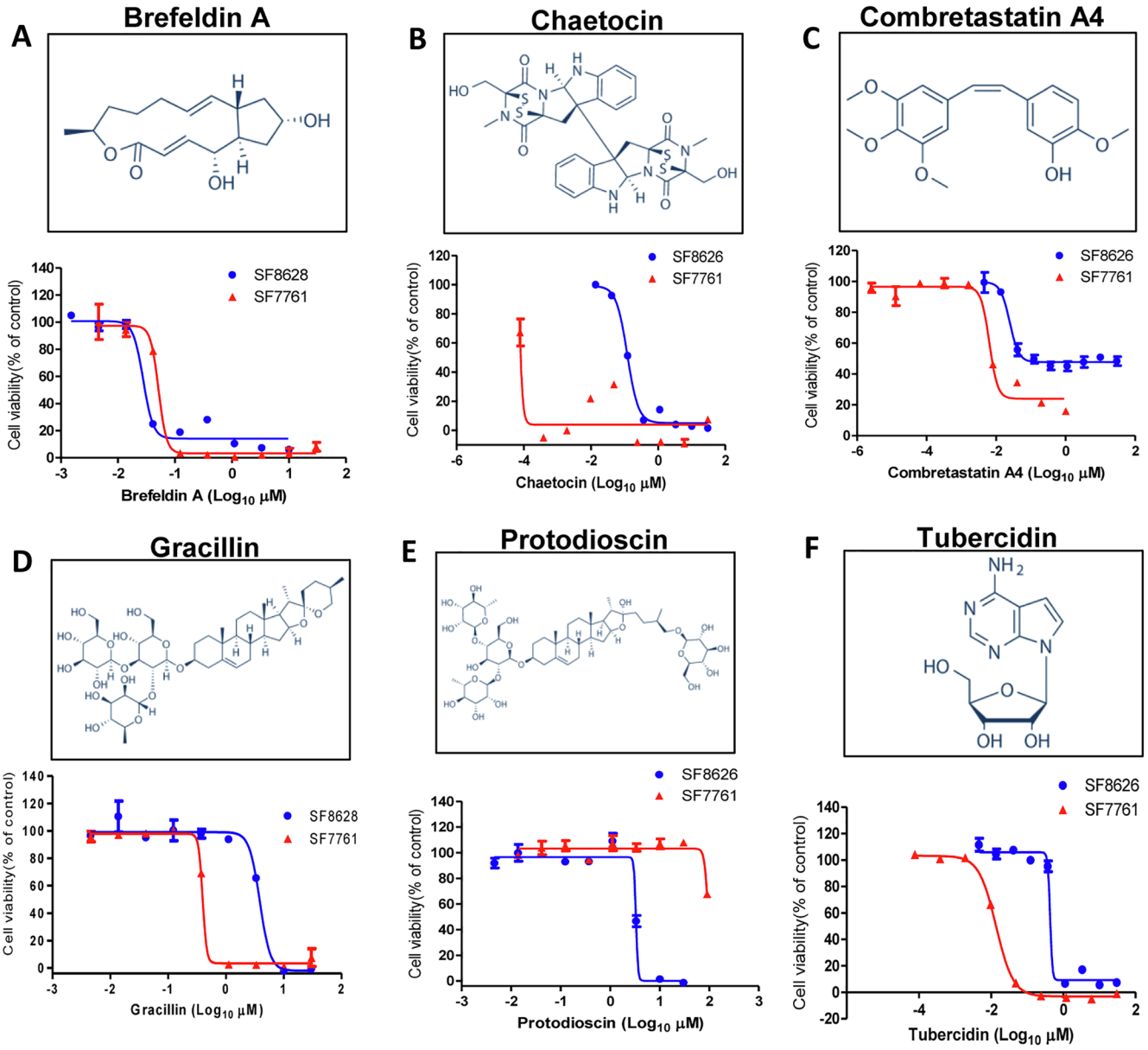


**Fig. 2.** The final hit compounds displaying inhibitory effects on DIPG cell growth. (A-F) The structures of the final 6 hit compounds and their dose-dependent "killing curves" on 2 DIPG cell lines, SF8628 and SF7761. Error bars represent S.D. for 3 independent experiments.

### 2.7. qRT-PCR

Total cellular RNA was isolated and purified using the RNeasy Mini kit (Qiagen). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen). Primers used for amplification of target genes were listed in Table S1. Amplification was performed on an iCycler IQ Real-Time PCR Detection System and analyzed as described previously [10].

### 2.8. Western blot

Total cell lysates (20 μg) were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies to phosphor (p)-PI3K/total (t)-PI3K, p-Akt/*t*-Akt, p-AMPKα/t-AMPKα, p-p65/t-p65 and GAPDH as a loading control (Cell Signaling). Immunoreactive bands were identified using an enhanced chemiluminescence reaction (Perkin-Elmer), and visualized by autoradiography.

### 2.9. Statistical analysis

Significant differences between experimental and control groups were determined using the two-tailed Student's *t*-test (Excel 2016).

## 3. Results

### 3.1. High-throughput screening and identification of new natural compounds with anti-DIPG activities

Currently, there are limited patient-derived DIPG cell lines commercially available for research use. One commercial DIPG cell line, SF8628, derived by surgical biopsy from an H3.3K27M DIPG patient [11] was used for our screening assays. H3.3K27M, a somatic mutation of histone H3.3 resulting in a lysine 27 to methionine substitution occurs in 60% of DIPG [12], which is thought to be important factor driving DIPG oncogenesis [12,13]. After screening a chemical library containing 756 natural products, we found 35 hit compounds

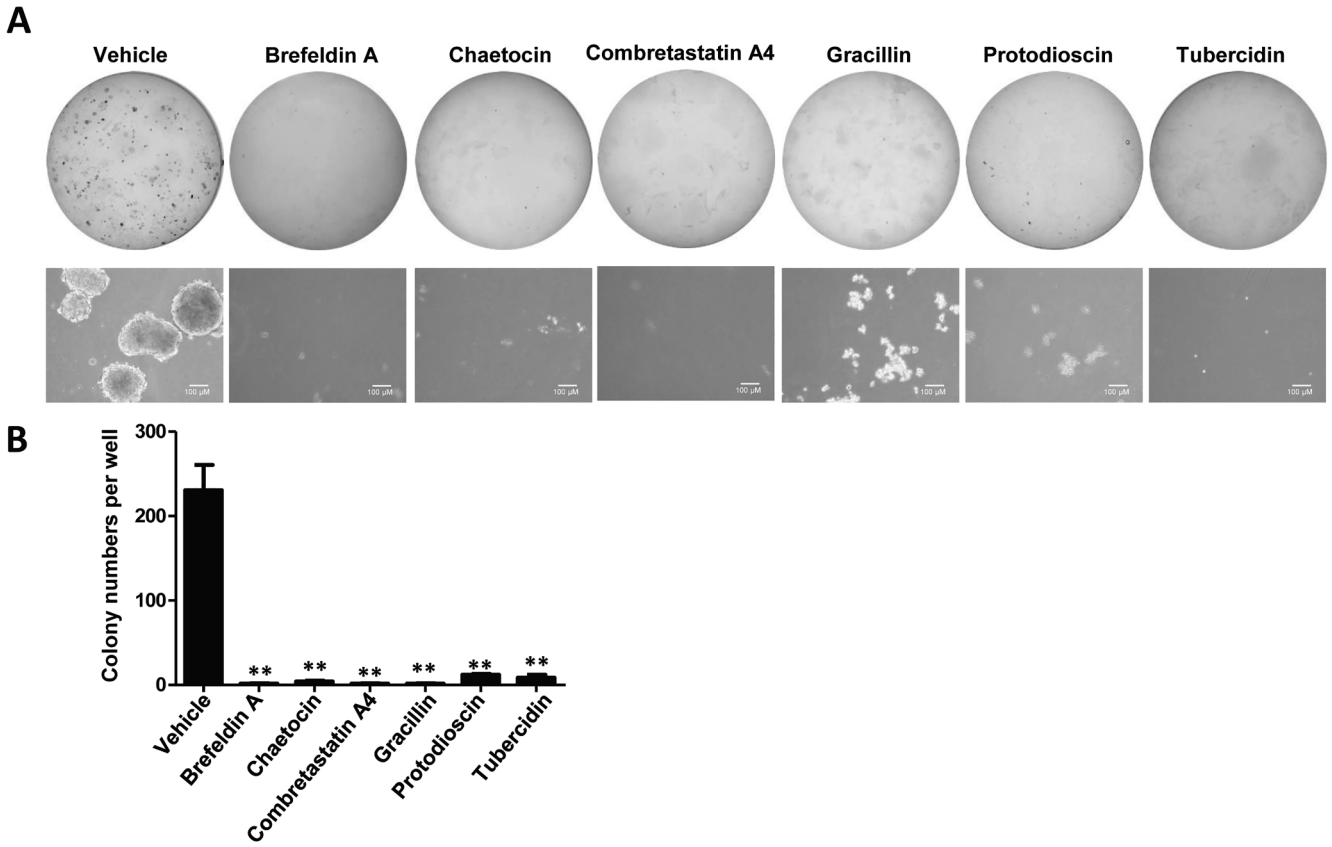


**Fig. 3.** The new natural compounds displaying inhibitory effects on DIPG anchorage-independent cell growth. (A and B) The inhibition of DIPG anchorage-independent growth ability by new natural compounds, using soft agar assays. Scale bars are 100 μm. Error bars represent S.D. for 3 independent experiments, ** = $p < 0.01$ (*vs* the Vehicle control).

displaying prominent cytotoxicity (> 60%) at 10 μM concentration (Fig. 1). These compounds were divided into several categories based on their putative targets: microtubules (7 compounds); DNA synthesis (9 compounds); protein synthesis (3 compounds); cell cycle (3 compounds); cellular signaling (9 compounds) and unknown targets (4 compounds) (Table S2). After searching published literature at PubMed and excluding those molecules with known anti-cancer activities (e.g., Paclitaxel, Docetaxel, Doxorubicin, Rapamycin), 8 hit compounds were left. After calculating the $CC_{50}$ using drug-killing curves on two DIPG cell lines, SF8628 and SF7761, we finally identified 6 hit compounds with $CC_{50} < 10$ μM (most of them at nM levels), including Brefeldin A (a lactone antibiotic), Chaetocin (a histone methyltransferase inhibitor), Combretastatin A4 (a microtubule-targeting agent), Gracillin (unknown target), Protodioscin (a testosterone inducer), Tubercidin (an adenosine analogue) (Table 1 and Fig. 2). Interestingly, we found that most of these compounds displayed better killing effects on SF7761 than on SF8628, except Protodioscin. Although both DIPG cell lines carry H3.3K27M, they display distinct morphology in cultures; SF7761 cell line exhibits much less adhesive capability, easy clustering, and difficultly being cultured (Fig. S1), so we decided to use the SF8628 cell line for subsequent experiments.

### 3.2. Explore the mechanisms of natural compounds "killing" DIPG cells using functional assays

We next sought to determine the mechanisms for anti-DIPG activities of these natural compounds. Using the soft agar assay, we observed that all these hit compounds dramatically inhibited anchorage-independent growth of DIPG cells when compared to the vehicle control (Fig. 3). The vehicle-treated DIPG cells usually formed big visible spheroids, in contrast, treatment with the hit compounds greatly shrank the spheroids or only left some cell debris in the wells. Using FITC-Annexin V/propidium iodide (PI) staining combined with flow cytometry detection, we found that most of these hit compounds significantly induced apoptosis of DIPG cells, including the increased subpopulation of both early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptotic cells when compared to the vehicle control (Fig. 4A and B). We also found that most of these compounds significantly caused DIPG cell cycle arrest, including the G1 or G2 phase arrest, especially the latter (Fig. 4C and D).

### 3.3. Transcriptomic analysis of gene profiling altered within natural compounds treated DIPG cells

To determine the global cellular changes induced by these 6 hit compounds, we compared the gene profile altered between vehicle- and compounds-treated tumor cells by using RNA-Sequencing analyses (Illumina). The volcano plots showed the numbers and scattering of genes were significantly upregulated or downregulated (≥2 folds and FDR < 0.05) in these hit compound-treated SF8628 cells, respectively (Fig. 5A). Intersection analysis (heat map) indicated that there were 64 transcripts, including some non-coding RNAs, which were commonly and significantly altered by at least 3 natural compounds treated DIPG cells (Fig. 5B). Notably, there were 4 transcripts commonly altered within all the 6 compounds treated tumor cells. Three of them were upregulated including DNAJC25-GNG10 (encoding a Guanine nucleotide-binding protein subunit gamma, ~5.0 folds averagely increased), RP11-1280N14.3 and RP11-323N12.5 (two of pseudogenes, ~100 and 220 folds averagely increased, respectively); while another pseudogene, RP3-430N8.11, was downregulated (~60 folds averagely decreased). GO_enrichment analysis of these common candidates indicated that several major functional categories were involved, especially the

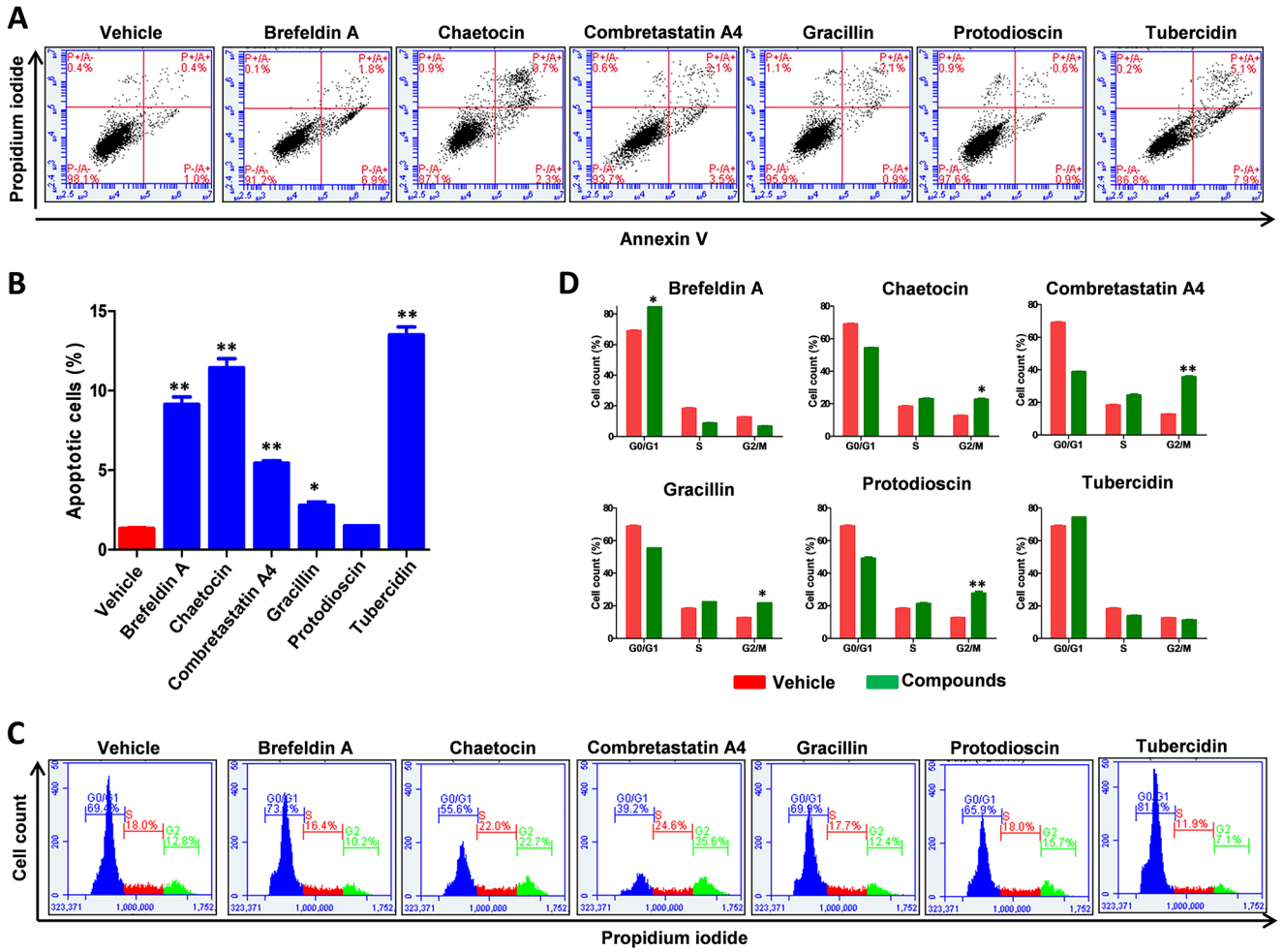


**Fig. 4.** The new natural compounds treatment induces DIPG cell apoptosis and cell cycle arrest. (A–D) SF8628 cells were treated with natural compounds or vehicle for 48 h, then cell apoptosis (A/B) and cell cycle (C/D) were measured by Annexin V-PI or PI staining and flow cytometry analysis. Error bars represent S.D. for 3 independent experiments, * = $p < 0.05$, ** = $p < 0.01$ (*vs* the Vehicle control).

regulation of cell or neuron death (Fig. 5C). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that 4 major pathways were affected which involve 6 genes commonly altered in these hit compounds treated DIPG cells (Fig. 5D and S2), implying that these pathways may have close association with DIPG survival and pathogenesis.

### 3.4. Identification of new cellular genes essential for DIPG survival

To functionally validate those significantly altered genes within most natural compounds treated DIPG cells discovered by RNA-Sequencing (especially those potentially related to tumor cell survival), we selected FN1 and EIF3CL, two downregulated candidates for understanding their roles in DIPG cell survival based on the following rationale. Fibronectin 1 (FN1), a member of the FN family, is involved in a variety of cellular processes and in diseases such as cancer, arthritis and atherosclerosis [14]. Furthermore, one recent study reported that silencing of the FN1 gene in human glioma cells inhibited cell proliferation, promoted cell apoptosis and senescence, and reduced cell migration and invasion through the disruption of the PI3K-Akt signaling pathway [15]. The functional role of Eukaryotic translation initiation factor 3 subunit C-like (EIF3CL), in cancer cells remains unknown, although other eukaryotic translation initiation factors (eIFs) have been extensively studied in a variety of cancers [16,17]. Here we found that successful knockdown of either FN1 or EIF3CL by RNAi effectively repressed DIPG cell proliferation and anchorage-independent growth, as well as induced tumor cell apoptosis (Figs. 6A–C and S3). To further explore the downstream signaling of FN1 and EIF3CL in DIPG cells, we found that silencing FN1 downregulated the activity of PI3K-Akt signaling, while silencing EIF3CL downregulated the phosphorylation of AMPKα and NF-κB p65 proteins (Fig. 6D). All of these signaling pathways have been reported in relation to cancer cell proliferation.

Currently, DIPG clinical database is not publically available. Recently, we have obtained the permission to access RNA-Sequencing data of DIPG tumor samples and paired normal brain tissues from the NIH Genotypes and Phenotypes (dbGaP) database. Using this database, we found that both FN1 and EIF3CL expression were higher in DIPG tumor samples than in the paired normal brain tissues, although only FN1 proved to be statistically significant (Fig. 6E). The lack of significance is likely due to the small number of samples available for analysis.

## 4. Discussion

In the current study, we for the first time identify promising natural compounds with anti-DIPG activities by high-throughput screening. These hit compounds have never been connected to DIPG treatment. One of the hit compounds, Brefeldin A, a lactone antiviral, was found to alter the morphology and function of the Golgi apparatus and endosomal compartments in different cell types [18]. One very recent study reports that Brefeldin A treatment can effectively prevent glioblastoma progression *in vivo*, which is not associated with the

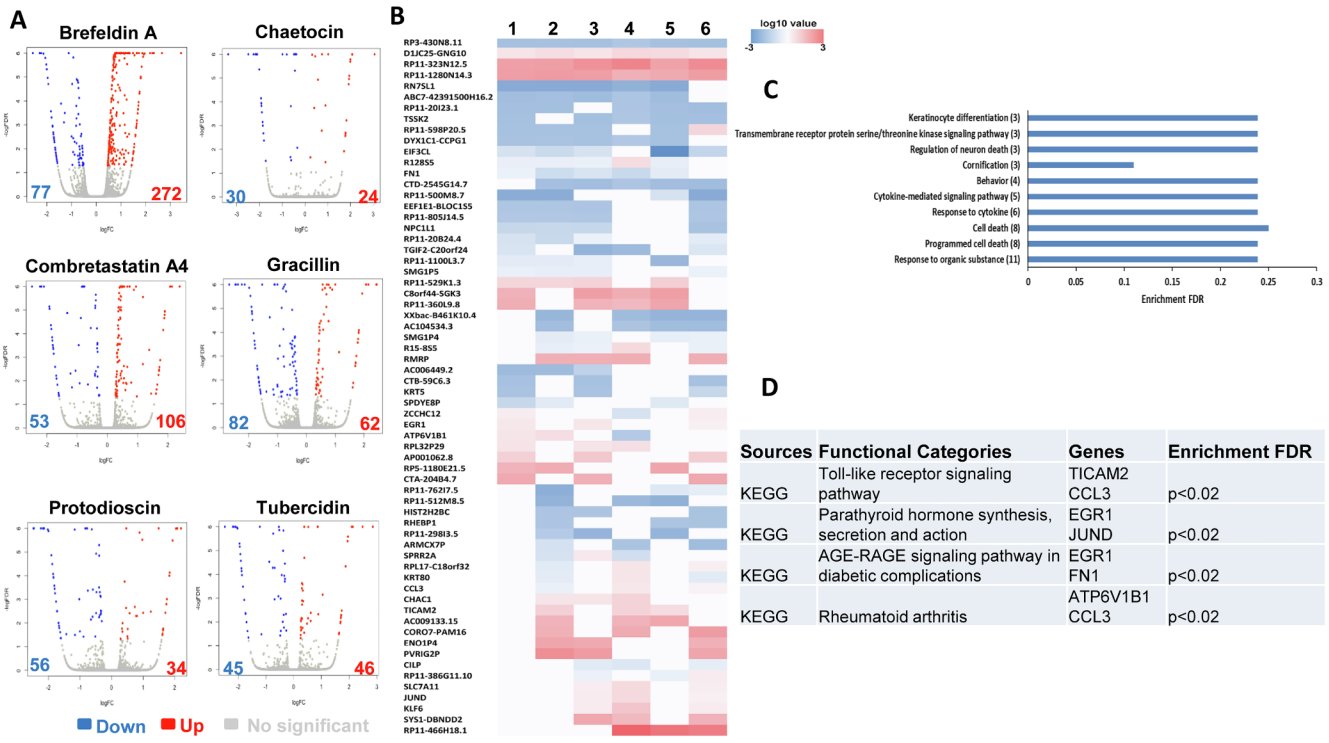


**Fig. 5.** Transcriptome analysis of new natural compounds treated DIPG cells. (A) The RNA-Sequencing (Illumina) was used to investigate the transcriptome change between natural compounds and vehicle treated DIPG SF8628 cells. The significantly changed genes (expression change ≥ 2-fold and $p < 0.05$) were shown in the Volcano plot panels. (B-C) The heat map and GO_enrichment analysis of commonly changed cellular genes by at least 3 natural compounds in DIPG. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of commonly changed cellular genes in DIPG.

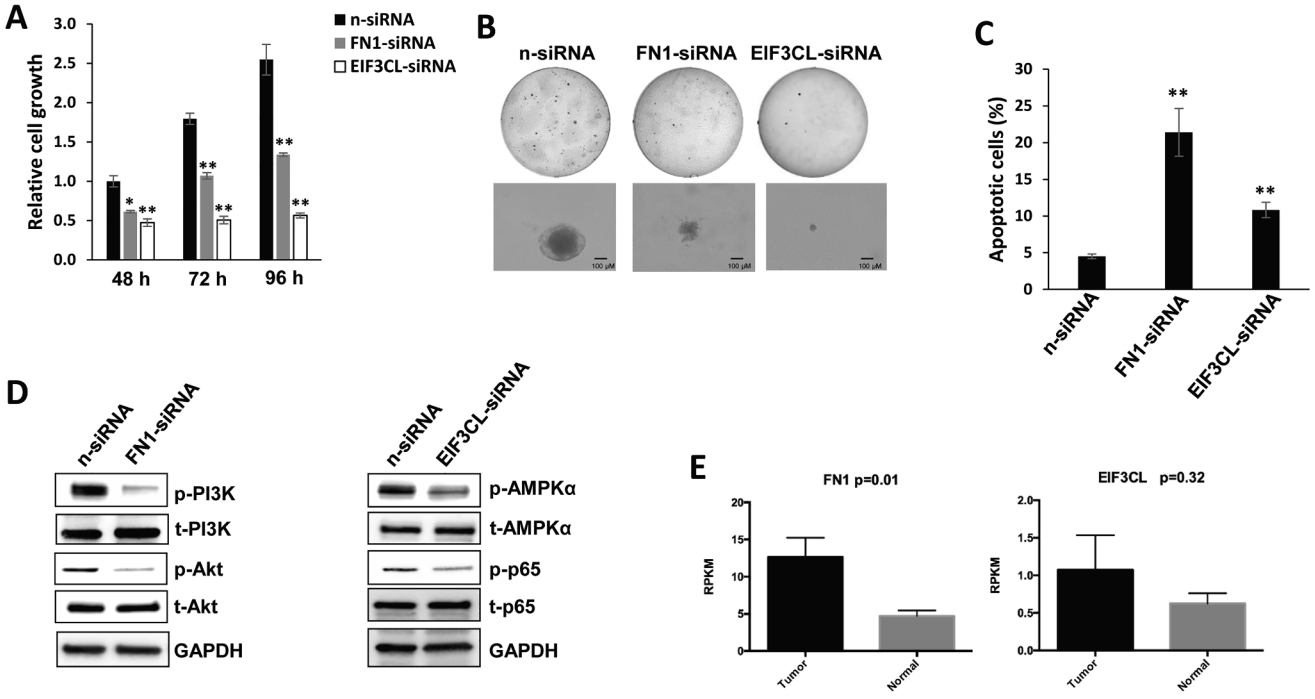


**Fig. 6.** Identification of new cellular genes required for DIPG cell survival. (A-D) SF8628 cells were transfected with *FN1*-siRNA, *EIF3CL*-siRNA or non-target control siRNA (*n*-siRNA) for 48–96 h, then the cell proliferation, anchorage-independent growth, cell apoptosis and protein expression were measured by using the WST-1 assays, soft agar assays, flow cytometry and Western blot analysis, respectively. Error bars represent S.D. for 3 independent experiments, ** = $p < 0.01$. (*vs* the *n*-siRNA control) (E) The expression of FN1 and EIF3CL in 10 DIPG and paired normal brain tissues. RPKM (Reads Per Kilobase of transcript per Million mapped reads) values were calculated using reads across the gene exons. Data were represented as the mean (+/-) SEM.

undesirable toxicity of Brefeldin A in other cancers (e.g., the Golgi disruption and the ER collapse) [19]. Another new study reports that Brefeldin A treatment can significantly inhibit self-renewal of glioma stem-like cells and tumor growth through downregulation of VEGF secretion [20]. Interestingly, Xu *et al* have reported that patient-derived DIPG cells preserve stem-like characteristics (e.g., the expression of

several neural stem cell lineage markers) and generate orthotopic tumors in mouse brainstem [21]. Therefore, we will further explore whether Brefeldin A treatment may affect these stem-like characteristics of patient-derived DIPG cells. Another hit compound, the fungal metabolite Chaetocin (also an inhibitor of histone methyl transferase SUV39H1) has been recently identified as a novel TRAIL (TNF Related Apoptosis Inducing Ligand) sensitizer to enhance TRAIL-induced glioblastoma apoptosis [22]. In addition, Chaetocin treatment has been found to regulate ATM-YAP1 and JNK signaling for the induction of ROS-mediated apoptosis in cancer cells [23].

Our transcriptomic analysis of gene profiling commonly altered within these hit compounds treated DIPG cells indicated that 3 pseudogene transcripts were dramatically upregulated or downregulated within all the 6 hit compounds treated tumor cells, while the functions of these pseudogenes in DIPG cells are unknown. Pseudogenes are evolutionally conserved and present in diverse organisms [24]. Interestingly, increasing evidence suggests that the dysregulation of pseudogenes is often associated with various human diseases including cancer [25]. Like other types of long non-coding RNAs (lncRNAs), pseudogenes can also function as master regulators for gene expression either positively or negatively. For example, PTEN pseudogene 1 (PTENP1) can function as decoys to adsorb microRNAs targeting the PTEN tumor suppressor for degradation [26]. On the opposite, suppression of the high mobility group A1 pseudogene (HMGA1P) mRNA results in a reciprocal increase in HMGA1 mRNA stability and expression levels [27].

During functional validation, we have identified FN1 and EIF3CL, two new cellular genes essential for DIPG survival. The functional role of EIF3CL in cancer cells has never been reported, although it has been found highly expressed in human glioma tissues through analysis of the Cancer Genome Atlas (TCGA) datasets when compared to other types of cancer (https://www.proteinatlas.org/ENSG00000205609-EIF3CL/pathology). We also found that both of genes had connection with several tumor cell proliferation associated signaling (e.g., PI3K-Akt, AMPKα and NF-κB), although the regulatory mechanisms remain unclear. In fact, it has been found that H3K27M co-occur with alterations in some signaling genes such as PI3K-Akt-mTOR to drive gene expression signatures and promote malignant transformation [28].

In summary, our data identified new cellular factors essential for DIPG survival and new promising natural compounds with anti-DIPG activities. Further studies will focus on their *in vivo* efficacy in DIPG animal models.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.103847.

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