Viability screen on pediatric low grade glioma cell lines unveils a novel anti-cancer drug of the steroid biosynthesis inhibitor family

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ABSTRACT

Pediatric low grade gliomas are the most common central nervous system tumors and are still incurable among a subset of patients despite current treatment modalities. Steroid biosynthesis occurs in a wide variety of organs including the brain, to mediate an assortment of functions, including a proposed role in the growth of gliomas. Hence, targeting steroid biosynthesis and/or their signaling pathways, is anticipated as an effective approach for treating gliomas. In this study, we investigated whether our chemical library of steroid inhibitors can modulate the growth of pediatric low grade glioma cell lines (Res186, Res259, R286), and subsequently identified a potent inhibitor of 17β-hydroxysteroid dehydrogenase type 3, referred to as DK16, which functions by attenuating cell viability, proliferation, migration/invasion and anchorage independent growth and conversely induces apoptosis and cell cycle arrest in a dose and duration dependent manner. Further investigations into the mechanisms of how DK16 functions showed that this drug increased the BAX/BCL2 expression ratio, induced phosphatidylserine externalization, and mitochondrial membrane depolarizations culminating to the release and nuclear translocation of AIF. In addition, treatments of low grade glioma cell lines with DK16 increased the expression of pro-apoptotic mediators including CDK2 and CTSL1, and with the converse diminished expression of pro-survival and migratory/invasion genes like PRKCA, TERT, MAPK8, MMP1 and MMP2. Our findings collectively demonstrate the potent anti-neoplastic properties of DK16, a steroid biosynthesis inhibitor, on the growth of pediatric low grade gliomas.

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1. Introduction

Pediatric low grade gliomas (LGGs) are the most prevalent form of childhood central nervous system (CNS) malignancies; representing a heterogeneous group of CNS neoplasms classified as World Health Organisation grades I and II tumors. Since 1879 following the successful surgical resection of meningeoma from a 14 year old girl by Scottish surgeon, Sir William Macewen, it became plausible that pediatric brain tumors are curable [1]. Even though surgery continues as the primordial treatment for low grade gliomas, it is still challenged by the infiltrative properties of some low grade tumors and those at risk of malignant progression [1]. In fact, these limitations in surgical interventions are further supported by a multicentre study showing gross total resection was only possible for 5/198 patients with low grade chiasmatic hypothalamic tumors [2]. Even after resection, tumor recurrence is still possible [3], with the 5 and 10 year progression free survival being 55% and 48% respectively after the initial surgery [4]. As a result, radiotherapy is sometimes routinely used as an adjuvant therapy in older children and adolescents, but is associated with increasing the risk of cognitive deficits, vasculopathy and tumor recurrence [5,6]. If left untreated, low grade gliomas may progress into malignancy which is often associated with poor survival and a dismal prognosis. With the objective of improving prognosis, chemotherapy has become a useful option and is often combined with surgery and/or radiation for the adjuvant treatment of pediatric low grade gliomas. In fact, there were some encouraging results with the adjuvant use of chemotherapy with or without other therapies for treating low grade gliomas [7,8]. However, factors such as the progression free survival (PFS) were identified to vary significantly with age, type of treatment, histological grade and tumor location amongst other factors [1,9–12]. Collectively, these current limitations associated with surgery and radiation, and the fact that chemotherapy can actually augment progression-free survival when used alone or as an adjuvant, have...
promoted us to identify and characterize more therapeutically effective drugs for the treatment of pediatric low grade gliomas.

Given the recent postulations that steroids could modulate the growth of gliomas [13], an important group of compounds worth investigating will be those that likely perturb the activity of enzymes involved in the final synthesis of androgens and estrogens. Indeed, some compounds belonging to this category had demonstrated high efficacies for the treatment of hormone-dependent cancers [14]. Crucial in steroid biosynthesis is a group of enzymes referred to as the 17β-hydroxysteroid dehydrogenases (17β-HSDs), which modulates the final synthesis step leading to the production of testosterone and estradiol. Over the years, 17β-HSD3 has been a promising and excellent target for hormone dependent diseases [15–17]. 17β-HSD3 converts 4-androstene-3,17-dione into testosterone, which in the presence of 5x-reductase, is converted to dihydrotestosterone or transformed to estradiol by aromatase. The importance of targeting these enzymes involved in the production of testosterone, dihydrotestosterone and estradiol, originates from the fact that these steroids potently activate receptors such as EGFR, I-GFR, GRP30, ER and AR or their downstream signaling effectors like MAPK, PI3K and AKT [18–22]; which are key modulators of cell viability, proliferation, migration and apoptosis in gliomas and other cancers. In fact, an assortment of gene fusion and activating mutations in key members of the MAPK signaling cascade are prevalent in the majority of pediatric low grade gliomas [23]. Hence, therapeutically targeting the upstream activators, for instance estradiol, is justified as a logical approach to curb the growth of low grade gliomas.

Other studies have further shown that estradiol and testoster-
one increase the viability of glioma cell lines in vitro [24,25]. Likewise, estradiol can also promote the survival of gliblastoma in vivo [26]. In addition, by inhibiting the synthesis of estradiol with aromatase inhibitors like melatonin and tibolone [27–29], it is possible to abrogate the growth of gliomas and hence highlighting the importance of targeting steroid biosynthesis as an effective strategy to treat gliomas. Considering the therapeutic potentials of inhibitors of 17β-HSD3 in the treatment of hormone-dependent diseases such as prostate cancer [14] and given the fact that steroids could bolster glioma growth, it is therefore logical to presume that evaluating inhibitors of 17β-HSD3 in gliomas, may lead to identifying suitable compounds with anti-neoplastic properties. Previously, we synthesized and developed chemical libraries comprising of several inhibitors of 17β-HSD3 [30,31]. In this study, we examined whether our library of 17β-HSD3 inhibitors could abrogate the growth of low grade pediatric glioma cell lines. Through a chemical viability screen, we identified DK16 from our chemical library, as the most potent inhibitor of the growth of pediatric low grade gliomas, using a wide variety of in vitro assays. Tumorigenesis studies with our panel of pediatric low grade glioma cell lines in immuno-deficient mice were hindered by their lack of in vivo growth. However despite this, our data has effectively shown that DK16 functions by significantly decreasing the growth of gliomas in vitro via the modulation of a wide variety of cellular processes (viability, proliferation, cell cycle, migration, invasion, apoptosis and anchorage independent growth) and by altering the expression of genes involved in key cancer progression pathways.

2. Materials and method

2.1. Chemicals and reagents

The synthesis and development of compounds used in the chemical screen were described previously [30–33]. A stock solution was prepared by dissolving all compounds including temozolomide (TMZ) and carbustine (BCNU) in DMSO and stored at 4°C. All cell culture assays had a final DMSO concentration of less than 1%. EMEM, trypsin-EDTA and antibiotic-antimycotic, were obtained from Invitrogen and Fetal bovine serum (FBS) was received from Fisher Scientific.

2.2. Tissue culture

The human neuroprogenitor cell line (ResCell VM, Millipore) was grown on laminin coated plates containing ResCell neural stem cell maintenance medium (Millipore) supplemented with 20 ng/ml of bFGF and EGF. The normal human astrocyte cell line (NHA) was a gift from Dr. Hoke (John Hopkins University, USA), and cultured in DMEM medium containing 10% FBS supplemented with 1× Non-essential Amino Acids. Res186 (pilocytic astrocytoma) and Res259 (diffuse astrocytoma) cell lines were previously characterized by us and shown to express several antigenic markers of low grade glioma surgical lesions [34]. The pilocytic astrocytoma cell line R286 which has an increased expression of the multi-drug resistance gene ABCB1 in response to vinblastine treatment [35], was kindly donated by Dr. Michael Bobola (University of Washington, Seattle, WA, USA). All pediatric glioma cell lines were cultured in DMEM/Ham’s F12 medium containing 10% FBS and 1% antibiotic-antimycotic solution, and maintained together with the normal cell lines, at 37°C with 5% CO2 and 100% humidity.

2.3. Viability chemical screen

A library of chemical compounds previously synthesized in our laboratory, were assigned into different groups based on unique chemical and structural properties. Forty-two distinct compounds were randomly selected from these groups and assayed for viability using the Celltiter 96 aqueous one solution cell proliferation assay (Promega). Briefly, 2.0 × 104 cells were seeded in 96 well plates and incubated at 37°C for 24 h. The cells were treated with 10μM of each compound except TMZ and BCNU (positive controls) which were used at a concentration of 40 μM. Treated cells were incubated at 37°C for 24 h followed by the addition of the Celltiter 96 AQueous one solution-MTS (Promega). The cells were incubated for 2 h at 37°C, during which the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxoxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, is reduced to a colored for-
mazan product. The absorbance at 490 nm which is directly proportional to the number of living cells was obtained and plotted against different treatments. The most potent compound derived from the chemical screen was subjected to a dose and time dependent MTS assay. The percentage of cytotoxicity was calculated as [(absorbance of each treatment/absorbance of untreated) × 100] and plotted as a function of drug concentration.

2.4. Trypan blue viability assay

2.0 × 103 cells were seeded in 96 well plates for 24 h followed by the daily addition of desired concentrations of drugs in each well for a period of 5 days. Prior to analysis, the tissue culture medium was removed, the cells trypsinized and diluted with an equal volume of trypan blue. The numbers of viable and non-viable cells were visually counted with the aid of a compound microscope. The percent viability was calculated as [(viable/viable + dead) × 100], and plotted as a function of drug concentration.

2.5. Methylene blue viability assay

2.0 × 103 cells were seeded in 96 well plates and incubated for 24 h at 37°C. The cells were then subjected to daily treatments within a 5 day period. After removing the tissue culture medium, 0.1% methylene blue in 20% ethanol was added to each well. The plates were incubated at room temperature for 5 min, and then excessive methylene blue was removed by vacuum aspiration. The plates were then carefully washed with phosphate buffer (PBS) and the mean absorbance was measured at a wavelength of 650 nm. The viability index of untreated cells (negative control) was set to an arbitrary value of 100%.

2.6. BrdU ELISA proliferation assay

Proliferating cells were quantified using the BrdU Proliferation ELISA kit (Roche) as described by the manufacturer. Briefly, 2.0 × 103 cells were added to each well in a 96 well plate and incubated at 37°C for 24 h. This was followed by the daily addi-
tion of drugs at varying concentrations for a 5 day period. Prior to analysis, the cells were labeled with BrdU for 18 h to allow BrdU incorporation into the DNA of pro-
liferating cells, and then fixed for 30 min at room temperature with FixDenat solu-
tion and exposed to a BrdU-POD antibody for 90 min at room temperature. The cells were then washed 3X with PBS followed by addition of 100 μl substrate solution and incubated at room temperature to permit optimal color development. The mean absorbance values of treated samples were plotted relative to the untreated control; the latter set at an arbitrary value of 100%.

2.7. Wound healing assay

1.0 × 103 cells were seeded on six well tissue culture plates and were allowed to reach confluence. A pipette tip was then used to create a wound at the center of the plate and the distance separating the cells (initial distance) was measured. The tis-
ue culture medium was removed and the cells washed with Phosphate buffered saline (PBS). Fresh tissue culture medium with different drug doses was
administered and the wells documented with a compound microscope equipped with a digital camera (at 0 h). A second image (at 36 h) was taken as soon as cells in the untreated wells migrated and healed the wound. The % migratory index was calculated as \( \frac{\text{final distance}}{\text{initial distance}} \times 100 \).

### 2.8. Boyden chamber invasion assay

Migration through an extracellular matrix was performed as previously described [36]. Briefly, the Boyden chambers (8 μm pore size) were coated with matrigel (BD Biosciences) and \( 1 \times 10^5 \) cells in serum free medium containing DK16 at different doses were added to the upper chamber. The lower chamber was filled with 500 μl tissue culture medium containing 5 μg/ml fibronectin. The plates were incubated at 37°C overnight, and then the cells on the upper chamber were removed with cotton pad soaked in PBS. The cells on the bottom matrix were fixed with methanol, stained with 0.005% crystal violet in 70% ethanol and documented with a compound microscope and digital camera. The invasion index was calculated from five random fields, and with untreated samples set to an arbitrary value of 100%.

### 2.9. Cell cycle analysis

\( 1 \times 10^5 \) cells were seeded in six well plates and treated with 5 μM, 10 μM or 20 μM DK16 or 40 μM BCNU. Prior to analysis, the cells were trypsinized, and fixed in PBS containing 70% ethanol for 30 min at 4°C. The cells were centrifuged, the ethanol removed, and then the pellet was suspended in PBS containing 50 μg/ml propidium iodide and 40 U/ml RNase. Following incubation at room temperature for 30 min, the cells were subjected to flow cytometry analysis using the BD FACS Canto II software.

### 2.10. Quantification of apoptotic cells

Cells undergoing apoptosis were detected using the annexin V-FITC/propidium iodide double staining kit (Biovision). \( 2 \times 10^5 \) cells were cultured in six well plates and 24 h later, subjected to different daily treatments for five consecutive days. On days 1, 3 and 5, cells were trypsinized, centrifuged and the pellet suspended in annexin V binding buffer, followed by the addition of annexin-FITC and propidium iodide as described by the manufacturer (Biovision). The cells were incubated in the dark for 5 min and subjected to flow cytometry. Annexin-FITC positive cells and propidium iodide stained cells were quantified using the BD FACS Canto II software.

### 2.11. Measurement of mitochondrial membrane potential

Mitochondrial membrane permeability was measured using DioC6(3), a dye which can quantify alterations in the mitochondrial membrane potential [37]. \( 1 \times 10^5 \) cells were seeded in 24 well plates and incubated overnight at 37°C. The cells were treated with different drug concentrations and incubated for 48 h. The medium was removed, the cells trypsinized and then the pellet suspended in 40 nM DioC6(3) dissolved in PBS. Viable and non-viable cell fractions were analyzed by flow cytometry using the BD FACS Canto II software.

### 2.12. Soft agarose assay anchorage independent growth

The bottom agarose layer was constructed from a 1:2.1 mixture of heated 1.8% agarose and tissue culture medium at 42°C. This mixture was transferred into 24 well plates and allowed to solidify at room temperature. The top agarose containing a 1:1 mixture of 0.8% heated low melting agarose at 42°C and \( 5 \times 10^5 \) cells in tissue culture medium, was then added onto the bottom agarose layer and allowed to solidify at room temperature. 100 μl of tissue culture medium was added to each well and the cells subjected to daily treatments with vehicle, DK16 or BCNU. The tissue culture medium was changed twice weekly and after 18 days of treatment, colonies were stained with 0.005% crystal violet and documented with a Nikon microscope equipped with a camera. Colonies were scored from five randomly selected fields of each sample tested.

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**Fig. 1.** Effect of DK16 on cell viability. (A) Cytotoxic evaluation of selected compounds numbered 1–42 using the MTS assay. Untreated (NT) and vehicle (Veh) represent negative controls whereas temozolomide (TMZ) and carmustine (BCNU) were used as positive controls. Since BCNU was more cytotoxic than TMZ, we focused on compounds with greater cytotoxicity than BCNU, and identified DK16 was the most potent among all three low grade glioma cell lines studied. Dose dependent decrease in viability of glioma cell lines following administration of varying doses of DK16 as determined by (B) MTS cytotoxicity assay; (C) Trypan Blue exclusion test; and (D) Methylene blue viability staining.
2.13. Real time reverse transcription (RT) PCR

Gene specific primers designed with Primer blast (NCBI) and the target sequences were chemically synthesized and then used to construct a 96 well PCR cancer pathway finder array. Each array consisted of six PCR quality control wells, four housekeeping genes and 84 carefully selected genes implicated in cancer progression and drug metabolism. A single dose of 10 μM DK16 was administered to cell lines in culture, prior to total RNA extraction after 3 h and 24 h, using the GenElute mammalian total RNA extraction kit (Sigma), as described by the manufacturer. cDNA was synthesized from 1 μg of total RNA using the Superscript II reverse transcriptase kit (Invitrogen). The cDNA was subjected to a 1:4 dilution in sterile distilled water and analyzed with the Ssofast Evagreen supermix (Biorad) using the CFX96 real time PCR detection system (Biorad). ACTB, GAPDH, B2 M and RPL13A were used as reference genes. Comparative normalized gene expressions were computed manually and validated using the SABiosciences PCR analysis tool (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Genes with Ct values >35 in untreated samples, were excluded from the analyses.

2.14. Evaluation AIF release from the mitochondria

The mitochondrial and cytosolic fractions of cultured cells were isolated using the mitochondria isolation kit (Thermos Fisher), as described by the manufacturer. Lysates from both fractions were subjected to Western blot analyses as previously described [34], and using the mouse monoclonal IgG2b antibody (E-1) (Santa Cruz Biotechnology) which can bind to the human mitochondrial apoptosis inducing factor (AIF). AIF nuclear translocation was examined on cells grown on microscope slides by Immunofluorescent cytochemistry. Briefly, cells were fixed with 20 °C ice-cold Methanol, and blocked with 0.1% Western Blocking reagent (Roche). These cells were then incubated with a 1:100 dilution of the AIF E-1 primary antibody (Santa Cruz Biotechnology), followed by secondary detection with Protein-G FITC. The nuclei were stained with 1 μg/ml Propidium iodide and then incubated with the Vectashield Hardset mounting medium. Images were documented with a Nikon 80i eclipse microscope and digital camera.

2.15. Statistical analysis

All experiments were done in triplicate. Results of replicate experiments were analyzed using the Graph Pad Prism Software and multivariate comparisons were performed using the ANOVA and Bonferroni post hoc tests. Standard errors were calculated from the mean ± SEM from replicate experiments and with a P-value < 0.05 considered as being significant.

3. Results

3.1. Chemical genetic screen identifies DK16 as a potent inhibitor of cell viability

To identify compounds which affect the viability of our panel of pediatric low grade glioma cell lines, we selected 42 compounds from our drug library for 24 h viability tests using the Celltite 96 aqueous one cell proliferation kit. Since BCNU (40 μM) was more potent than temozolomide (TMZ) (40 μM) in inhibiting the growth of our panel of pediatric low grade glioma cell lines, we established the cut-off absorbance baseline value from BCNU to identify more effective cytotoxic compounds (Fig. 1A). A vast number of compounds was discovered as being more potent than BCNU in inhibiting the viability of the diffuse infiltrating astrocytoma cell line (Res259), and included compounds 1, 2, 4, 7–17, 20, 22–26, 28, 30–40 and 42. For the pilocytic astrocytoma cell lines Res186 and R286, compounds 7, 10, 11, 12, 16–18, 31, 32, 33 39 were observed to be more effective than BCNU (Fig. 1A). Only compound 16 consistently inhibited the viability of all three glioma cell lines most effectively with >60% reduction in viability within 24 h. Compound 16 was previously reported by us as compound 15b (3α,5α)-

![Fig. 2. Effect of DK16 on cell proliferation and cell cycle. (A) Dose dependent decrease in BrdU stained cells following administrations of 5 μM, 10 μM and 20 μM of DK16 within a 5 day period. (B) Cell cycle analyses of DK16 treated and untreated cells. Cells treated with DK16 showed gradual accumulation within the S and G2 M phases of the cell cycle compared to the untreated cells.](image-url)
3-\{[\text{trans-2,5-dimethyl-4-\{[2-(trifluoromethyl) phenyl sulfonyl]} piperazin-1-yl\} methyl]-3-hydroxyandrostan-17-one\} [30] and as the most potent 17β-HSD3 inhibitor in HEK-293 transformed cells. In this study we designate this compound as drug DK16. Although the vast number of compounds included in our chemical screen perturbed the viability of pediatric glioma cell lines, as judged from the MTS assay, a few chemical agents like compounds 18, 27, 29, 36 and 37, were capable of promoting an increase in metabolic activity. This could potentially be a consequence of many reasons, including the ability of a compound to modulate the cytoplasmic volume, cellular physiology or metabolic activities, thereby augmenting the cellular levels of NADH or NADPH. In the MTS assay, this can subsequently increase the bio-reduction of tetrazolium salts and the production of higher levels of formazan. In order to circumvent these limitations of the MTS assay, we pursued additional methods to assess the effect of DK16 on cell viability (Fig. 1C and D).

Using the MTS assay, we next investigated whether DK16 can maintain a consistent reduction in cell viability over a 5 day period. Within the first 24 h, Res259 and R286 were only slightly responsive to 5 μM of DK16. On the contrary, administration of 10 μM and 20 μM DK16 to all cell lines resulted in a significant dose dependent decrease in cell viability (P-value < 0.0001) compared to untreated or vehicle treated cells during the 5 day period (Fig. 1B). These results were further confirmed with the trypan blue exclusion test and the methylene blue viability assay (Fig. 1C and D). The IC_{50} of DK16 was calculated as 4.9 μM, 9.1 μM and 9.5 μM in Res186, Res259 and R286 respectively.

Finally, we examined the effect of DK16 on the viability of two non-transformed or normal cells of the nervous system, namely, human astrocytes and neuroprogenitors. Interestingly, the administration of DK16 only modestly retarded the viability of a human non-transformed astrocyte cell line (NHA) compared to the negative controls (Supplemental Fig. 1A). Such phenomenon was only evident by day 5. On the contrary, DK16 failed to perturb the viability of the human neuroprogenitor cell line (RenCell VM) (Supplemental Fig. 1B). Hence, the minimal cytotoxic properties of DK16 on non-transformed or normal cells, makes it an attractive chemical agent for further investigations as a potent anti-neoplastic compound for the treatment of gliomas.

### 3.2. DK16 decreases proliferation and modifies the cell cycle

To investigate the mechanism of how DK16 interferes with cell viability, we first utilized the BrdU ELISA based proliferation assay. In contrast to the diffuse astrocytoma cell line Res259 [34] which...
doubles every 24 h, the pilocytic astrocytoma cell line Res 186 doubles every 48 h [34]. We deduced a similar doubling time of 48 h for the R286 pilocytic astrocytoma cell line. Despite these differences in doubling times, DK16 at 5, 10 or 20 μM significantly induced >60% decrease in proliferating Res259 and R286 cells within 24 h compared to the negative controls (P-value < 0.001). Moreover, further daily treatments significantly induced >80% decrease in proliferation within 72 h in comparison to the negative controls (P-value < 0.001). The Res186 cell line required a higher DK16 dose of either 10 μM or 20 μM to induce similar effects (Fig. 2A). Since DK16 influences the proliferation of glioma cell lines, we next mechanistically investigated whether the cell cycle is also affected. Subsequent cell cycle analyses revealed a significant progressive accumulation of cells in the S and G2 M phases with DK16 treatments, in a dose dependent manner within a 5 day period (Fig. 2B).

3.3. DK16 induces apoptosis

Since our precursory cell cycle analyses of cells treated with DK16 significantly demonstrated a dose and time dependent increase of cells in the sub-G₁ phase compared to the negative controls (P-value < 0.0001) (Fig. 3A), we further mechanistically investigated the effect of DK16 on apoptosis. Indeed our experiments with Annexin-V-FITC/PI staining, revealed a significant dose and time dependent increase in the accumulation of low grade glioma cells having the translocated phosphatidyl serine (indicative of early apoptotic cells) and as well as increased propidium iodide stained cells (indicative of late apoptotic cells) (Fig. 3B). In fact, by augmenting the dose (5 μM to 10 μM) and duration (1–5 days) of DK16 treatments, there were significant increases in the proportion of apoptotic and necrotic cells, except for Res186 which had instead a modest borderline insignificant change within the first 24 h, but with significant changes (P-value < 0.0001) becoming more prominent by the fifth day (Fig. 3B).

We then further investigated the effect of DK16 on the mitochondrial membrane potentials. Changes in the mitochondrial membrane potential (ΔΨₘ) are a prerequisite of the intrinsic apoptotic pathway. Cells stained with the Dioc6(3) dye selectively targets mitochondria with a compromised membrane [32]. After 48 h of DK16 treatments of varying doses, flow cytometric analyses revealed a significant dose dependent increase in Dioc6(3) stained
3.4. DK16 mediated apoptosis is associated with mitochondrial release and nuclear relocation of AIF

To evaluate whether DK16 mediated induction of BAX/BCL2 ratio and the mitochondria dissipation was associated with the release of mitochondria cell death mediators such as the Apoptosis Inducing Factor (AIF), we examined the mitochondrial and cytosolic fractions from time course experiments. DK16 induced a time-dependent release of AIF from the mitochondrial fraction to the cytosolic fraction in Res259 cells, and which peaked by 48 h (Supplemental Fig. 2A). This is contrary to untreated cells that instead expressed high levels of the mitochondrial AIF but not cytosolic AIF within the same period (Supplemental Fig. 2A). Immunofluorescent cytochemical analyses of the Res259 cell line further showed that cells treated with DK16 could induce the perinuclear re-localization of AIF (Supplemental Fig. 2B), and the translocation into the nucleus with concomitant nuclear condensation and DNA fragmentation (Supplemental Fig. 2C). Collectively, these data are indicative of the induction of apoptosis following treatments of pediatric glioma cell lines with DK16.

3.5. DK16 blocks migration and invasion of glioma cells

Gross total surgical resection of diffuse low grade astrocytomas is often limited by the infiltrative property of the tumors. Therefore, we next mechanistically investigated whether DK influences the migratory/invasive properties of glioma cells. Scratch assays showed a significant decrease (P-value < 0.0001) in the migratory potential of our panel of glioma cell lines treated with 10 μM or 20 μM of DK16 compared to untreated or vehicle treated cells (Fig. 5A). Subsequent studies using the Boyden Chamber invasion assays indicated similar findings with a significant dose dependent inhibition of invasion of our panel of glioma cell lines (Fig. 5B).

3.6. DK16 inhibits anchorage independent growth

The decreases in glioma cell viability and proliferation with DK16 treatments were further recapitulated by in vitro soft agarose transformation experiments, which demonstrated significant dose dependent decreases in the sizes and numbers of soft agarose colonies compared to the negative controls (Fig. 5D). Our tests showed that DK16 can efficiently cross the blood brain barrier (data not shown). However, since the panel of low grade glioma cell lines used in this study could not grow in vivo in immunodeficient mice, we could not further pursue in vivo transformation assays with DK16.

3.7. DK16 perturbs the expression of key genes involved in cancer progression

To understand the molecular mechanism of how DK16 mediates its functions, we performed real time PCR expression studies on a home-made Cancer Pathway Finder array comprising of four housekeeping genes and 84 genes which have functions in apoptosis, proliferation, migration and invasion, cell cycle, transformation, drug metabolism, DNA damage and repair. Subsequent analyses of genes expressed at three and 24 h post-treated with DK16 revealed 26 genes with significant differential expression of greater than two fold increased or decreased in normalized gene expression.

Fig. 5. Effect of DK16 on cell migration and invasion. (A) Migration index deduced after a 36 h wound healing assay and (B) Extent of invasiveness following treatments of glioma cell lines with varying doses of DK16 for 24 h, using the Boyden chamber invasion assay. (C) Representative images of triplicate wound healing experiments. (D) Inhibition of anchorage independent growth of glioma cell lines by DK16. Clonogenic survival of glioma cell lines cultured in soft agarose and with daily treatments of varying doses of DK16 for 18 days. DK16 treated cells showed a decline in the number of colonies relative to untreated or cells treated with PBS (vehicle). Error bars represent mean ± SEM of triplicate experiments.
expression (Fig. 6A and B). For instance, within the first 3 h post-treatment with DK16, the cell cycle transcriptional regulator CDK7 and pro-survival mediators GPX1, MAP2K6 and MAPK8 had greater than two fold decreased expression. In fact, this finding is therapeutically significant since gene fusions between KIAA1549 and BRAF and aberrancies among members of the MAPK signaling pathway are common in pediatric low grade gliomas [23]. Similarly, MMP1, MMP2, hTERT, PRKCA, TUBA1A expression begins to decline at 3 h, and with maximal decrease at 24 h. Contrary, RAD21, NFkB and ALFAM1 progressively increased expression from 3 to 24 h. The expression levels of these key cancer progression genes correlated with the in vitro properties of our panel of pediatric low grade glioma cell lines.

4. Discussion

Targeting steroid biosynthesis is becoming eminent as an important therapeutic strategy for the treatment of gliomas [13,25,38]. Indeed, 17β-HSDs were previously demonstrated by several studies as effective targets in tumors originating from steroidogenic tissues [14,38–40]. Like these steroidogenic tissues, gliomas also express pivotal enzymes and signaling pathways involved in steroid biosynthesis, including aromatase [41] and 5α-reductase [42] which catalyzes the conversion of testosterone to estradiol and dihydrotestosterone. Most importantly, prior studies demonstrated the potent influence of testosterone and estradiol on the growth of gliomas [24–26]. Depending on the cellular physiology, both dihydrotestosterone and estradiol can induce the activation of the classic estrogen and androgen receptors, as well as other growth promoting receptors like EGFR and I-GFR and their cascades of downstream signaling effectors including PKA, PKC, MAPK, Raf, ERK, PI3K and Akt; many of which are implicated in driving cancer progression [18–22,43]. Tamoxifen which is also clinically used as an adjuvant drug for the treatment of gliomas, competes with estradiol to bind to the estrogen receptor [44] and subsequently only inhibits the function of this receptor and reduces the production of estradiol.

However, the inhibition of 17β-HSD3 is anticipated to reduce the production of testosterone resulting in reduced levels of its metabolites estradiol and dihydrotestosterone, which is a better strategy in diminishing the neoplastic promoting properties of steroid metabolites. It is therefore imperative to investigate other compounds which target steroid biosynthesis, such as via 17β-HSD3, which can be more clinically effective in the treatment of gliomas.

Using a chemical viability screen, we identified several steroid inhibitors affecting the growth of our panel of pediatric low grade glioma cell lines among which DK16 (an inhibitor of 17β-HSD3) was the most potent compound with a calculated IC50 value being 4.9–9.5 μM. DK16 can also effectively cross the blood brain barrier, hence demonstrating its potential clinical usefulness. Our pre-clinical studies collectively indicate that DK16 mechanistically functions in glioma cell lines, by significantly decreasing cell viability, proliferation, migration/invasion and anchorage independent growth, and with the converse induction of apoptosis; in a drug dose and duration dependent manner. These physiological changes

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Fig. 6. Effect of DK16 on gene expression. (A) Clustergram depicting changes in gene expression at 0 h (NT), 3 h and 24 h after treatments with DK16 in Res259. (B) Alteration of gene expression following treatment of Res259 cells with a single 10 μM dose of DK16 for 3 h and 24 h. Shown are genes having >2-fold increased or decreased expression (P-value < 0.05). Negative values represent genes with decreased expression, whereas positive values are genes with increased expression.
are correlated with the modulation of relevant cancer progression genes by the decrease in expression of pro-survival and migration/invasion genes and the converse increase in expression of pro-apoptotic genes.

In fact in pediatric low grade glioma cell lines, DK16 dose dependently induced phosphatidylinerine externalization and the dissipation of the mitochondrial membrane, which were mechanistically linked to pro-apoptotic molecular events. These findings were further supported by the sustained increased expression of BAX but not BCL2 (as judged from the increased BAX/BCL2 ratio), and as well as in the increased expressions of CDK1, CDK2 and CTS1; all being important mediators of apoptosis [45–47]. While the increase in CDK2 activity is functionally linked to the loss of mitochondrial membrane potential [46], the lysosomal cathepsins CTS1, which mediate the cleavage of pro-apoptotic Bid and anti-apoptotic MCL1, BCL2 and BCL-XL [47], can significantly contribute to mitochondrial depolarization, leading to the release of proteins like cytochrome c, endo G and AIF. In accordance with these mechanistic events, we identified a 28-fold increased expression of the mitochondrial apoptosis inducing factor (AIFM1) gene within 3 h of treatment with DK16, and a further 49-fold increase in expression by 24 h. DK16 mediated mitochondrial depolarization was associated with the release and translocation of AIF to nucleus. AIFM1 functions as a major effector of the intrinsic apoptosis pathway and its depletion from the mitochondrial intermembrane space abrogates nuclear apoptosis in in vitro studies [48]. Once released into the cytosol, AIFM1 promotes pro-apoptotic activities including, phosphatidyl serine exposure on the plasma membrane, cytochrome c and procaspase 9 releases from the mitochondrial, followed by nuclear translocation leading to chromatin condensation and DNA fragmentation [48]. This is consistent with our finding in the panel of glioma cell lines treated with DK16 that showed evidence of atypical nuclei morphologies similar to those of apoptotic cells (data not shown). Furthermore, the pro-apoptotic functions of DK16 correlates with decreased expression of key cancer progression mediators of cell migration and growth signaling including PRKCA, hTERT, MMP1 and MMP2 [49–52]; suggesting a likely mechanistic explanation of why a significant fraction of DK16 treated cells inhibited cell migration/invasion in our scratch and boyden chamber matrigel assays. Indeed, an abrogation of cell migration could also be mechanistically caused by a proportion of cells choosing to enter cell cycle arrest or into apoptosis as judged from the evidence.

In summary, we report the drug – DK16, an inhibitor of 17β-HSD3, can modulate the expression of several oncogenic mediators leading to decreased cell viability, proliferation and migration/invasion, and the induction of growth arrest and apoptosis. Despite the lack of readily available xenograft models from our panel of pediatric low grade glioma cell lines for in vivo evaluations, our research has identified DK16 to efficiently cross the blood brain barrier and can significantly inhibit anchorage independent growth. Collectively, these robust pre-clinical findings extend promising results on the potent anti-neoplastic properties of DK16 in the treatment of pediatric low grade gliomas.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.11.034.

References
