TARGETING THE SWEET TOOTH OF TUMORS

Martyn A. Sharpe, David S. Baskin, Alexandra M. Baskin & Brianna N. Baskin

Kenneth R. Peak Brain & Pituitary Tumor Center
DISCLOSURES

NO DISCLOSURES
Introduction

It has been previously noted that gliomas highly express a range of sugar transporters to fuel their both catabolic (‘breaking down’) and anabolic (‘building up’) metabolism.

Using the TCGA database we were able to identify that upregulation of enzymes involved in galactose metabolism, with respect to the usage of galactose in glycan synthesis, were linked to poor patient survival.

We show that in addition to the previously identified upregulation of the glucose/galactose transporter Glut3, GBM also up regulates the normally testicular-specific glucose/galactose transporter Glut14.

As GBM will be the main tissue that has high galactose importer activity, we therefore investigated the use of a galactose anti-metabolite, 4-deoxy, 4-fluoro-galactose (4DFG), to disrupt the ability of GBM to synthesize glycans.

4DFG has been shown to be a competitive inhibitor of UDP-glucose 6-dehydrogenase (UGDH): a cytosolic enzyme that converts UDP-glucose to UDP-glucuronate. 4DFG has been shown to be a competitive inhibitor of UDP-galactose 4-epimerase (GALE): a epimerase which catalyzes the reversible conversion of UDP-galactose to UDP-glucose.

We show that 4DFG disrupts the normal glycan synthesis of GBM in vitro. We show that 4DFG lowers GBM viability and growth in vitro. Finally we demonstrate in a mouse flank model of primary GBM that 4DFG is able to arrest tumor growth.
Methods

**Tumor Microarray**: Gliomal tumors were taken at the time of excision and given a laboratory ID. Pieces >400 mg were fixed in PFA and a tumor microarray was generated consisting of normal human brain tissue, 8 low grade glioma and 21 GBM. The tissue microarray block was sliced into 5-μm sections that were affixed to slides and dried. Slides were dewaxed four times in xylene, twice in isopropanol, and rehydrated using graded ethanol. The slides were washed and permabilized using PBS containing 0.1% TritonX-100. Epitope retrieval was performed by heating the slides in Na-citrate buffer (100 mM, pH 6.0) at < 100°C in a domestic vegetable steamer for 30 minutes. After washing in PBS, endogenous peroxidase activity was eliminated using mild conditions: 1.8% H$_2$O$_2$ for 5 minutes, 1% sodium periodate for 5 minutes, 0.02% NaBH$_4$ for 2 minutes. The slides were blocked using Serum-Free Protein Block (Dako North America, Inc., Carpinteria, CA, USA) and incubated with primary antibodies overnight at room temperature, at 1:100 dilution. After washing in PBS, the HiDefTM HRP-polymer system (Cell Marque, Rocklin, CA, USA) was used to functionalize with peroxidase, and visualization was performed using the Dako DAB chromogen kit according to manufacturer’s guidelines. Slides used for quantification were treated with DAPI and visualized using fluorescence microscopy to identify non-necrotic areas of the tumors. Additional slides were also incubated with hematoxylin/100 mM LiOH to render the nuclei blue for visualization of cells using transmission microscopy.

Glioma cells and normal human astrocytes (NHA) were fixed in ice-cold 4% PFA and were washed/permeabilized with PBS with 0.1% Triton X-100 and treated with FITC-labeled lectins (EY Laboratories, San Mateo, CA) previously described.

**Primary GBM cell-line**: A GBM tumor was collected and washed in Phosphate Buffered Saline (PBS, Fisher Scientific, Waltham, MA), chopped with a scalpel and homogenized in a BeadBug™ homogenizer (Benchmark Scientific, Inc. Edison, NJ) using 1.5 mm Zirconium beads, in an equal volume of PBS. An aliquot was diluted 1:1 in Matrigel™ and injected into the flanks of two Balb/c mice. Tumors were passaged twice in Balb/c mice and then expanded in nude mice (NU-Foxn1$^{nu}$) for three passages, harvested, frozen and archived.

**Flank tumor model**: GBM flank tumors were generated and maintained, in nude mice (NU-Foxn1$^{nu}$) twice and a donor tumor (GBM157). **Cultured Cells**: Cells from the second aliquot of GBM157 homogenate were grown in Dulbecco’s modified Eagle’s medium (DMEM) with fetal bovine serum (FBS, 20%), 1U GlutaMax™, sodium pyruvate (1mM), penicillin (100U/ml), and streptomycin (100mg/ml). GBM157 cells are spontaneously immortal and were frozen at the fourth passage and used between seventh and ninth passages.

Glioma cells were grown to achieve confluency 24 hours after drug/vehicle dosing in Costar 96-well plates (Corning, NYC, NY, USA).

**Fluorescence microscopy**: Images were captured using a Nikon Eclipse TE2000-E at 4x, 20x or 30X magnification using a CoolSnap ES digital camera system (Roper Scientific) containing a CCD-1300-Y/HS 1392-1040 imaging array that is cooled by Peltier. Images were recorded and analyzed using Nikon NIS-Elements software (Elements 3.22.11). All images were saved as JPEG2000 files using Nikon NIS-Elements. The emission of FITC-labeled MGMT was collected using ex 450–490 nm, em 500–550 nm and Hoechst 33342 using ex 325–375 nm, em 435–485 nm.

**Cell viability**: Cells were incubated for 30 minutes with Hoechst 33342 (10µM) and then were fixed with ice-cold 4% paraformaldehyde. We conducted cell counts in center field at x4 or x20 magnification, depending on cell density. Dead/dying cells were identified as having condensed nuclei with signal intensities over threefold that of the median cell nuclei or being identified as fragmented.
Figure 1 shows the main pathway by which cells make and import UDP-galactose into the ER, for glycan synthesis. The galactose scavenging pathway uses either Glut3 or Glut14 (1), to import serum galactose. Table 1 shows that upregulation of either Glut3, Glut14 or both cuts patient survival by $\approx 6$ months. The galactose mutase (2) GALM is also a risk factor in GBM, and upregulation of either of the galactose kinases (GALK1&2; (3)) are risk factors for GBM patients.

The enzyme that carries out the interconversion of UDP-Glucose into UDP-Galactose (GALE (5)) is a risk factor for patients and is inhibited by 4DFG. The high levels of the endoplasmic reticulum UDP-Galactose importer, SLC35D2, are also a risk factor.

UGDH (7) converts UDP-Glucose to UDP-glucuronate (needed for the biosynthesis of the hyaluronan, chondroitin and heparan families of glycoproteins) is also inhibited by 4DFG.

Table 1 TCGA GBM: Effect of high expression of Galactose metabolism gene on GBM Patient outcome (low-high)

<table>
<thead>
<tr>
<th>Gene</th>
<th>GBM Patient $\Delta$ Months</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT3</td>
<td>6.15</td>
<td>0.0002</td>
</tr>
<tr>
<td>GLUT14</td>
<td>6.15</td>
<td>0.0113</td>
</tr>
<tr>
<td>GLUT3&amp;14</td>
<td>6.1</td>
<td>0.0036</td>
</tr>
<tr>
<td>GALM</td>
<td>6.3</td>
<td>0.021</td>
</tr>
<tr>
<td>GALK1</td>
<td>5.2</td>
<td>0.13</td>
</tr>
<tr>
<td>GALK2</td>
<td>5.7</td>
<td>0.06</td>
</tr>
<tr>
<td>GALK1&amp;2</td>
<td>5.7</td>
<td>0.01</td>
</tr>
<tr>
<td>GALT</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>GALE</td>
<td>8.5</td>
<td>0.0009</td>
</tr>
<tr>
<td>SLC35D2</td>
<td>6.20</td>
<td>0.05</td>
</tr>
<tr>
<td>UGDH</td>
<td>1.9</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Results:

Figure 2: GALT2 levels in low grade glioma and GBM

Figure 2 shows representative images of GALT2 levels in a range of gliomal tumors and in normal human brain. It is noted that GALT2 levels are highly upregulated and this is more pronounced in higher grade tumors.

Figure 3: Glut3 and Glut14 levels in Glioma

Figure 3 shows the levels of Glut3 and Glut14, normalized to normal human brain, in a range of gliomal tumor. The absorbance of $n=3$ different DAB visualized levels of these transporters was averaged and the error bars are the SEM. On average, the low grade gliomas have 5.2 times more Glut3&14 than control tissue and GMB more than 6.7 times the levels of GLUT3&14. It is noteworthy that (testicular)-Glut14 was expressed in tumors derived from both male and female patients.
Results: Glycan synthesis is altered in GBM cells incubated with 4DFG.

Figures 4A & 5A show images of astrocytes or GBM cells, labeled with either WGA or LPA lectins. The quantified levels per cell or per nuclei are shown in Figure 4B & 5B (n=8). It can be seen that both GalNAc-GalNAc and terminal sialic acid (UDP-Galactose derived) are similar to astrocytes. However, levels collapse when incubated with 4DFG.
Results: 4DFG inhibits growth \textit{in vitro} and \textit{in vivo}

Figures 6 shows that 4DFG causes a concentration and time dependent drop in living cell numbers in primary human GBM cells grown in culture ($n=6$, mean and SD). Statistical significance at $p<0.05$ is attained at concentrations of 120 $\mu$M and above, at 24 hours. When incubated for 48 hours cell numbers drop by 50% at concentrations above 150 $\mu$M (at the $p<0.001$ level) and cells have altered morphology and very large nuclei (see Figures 4&5A).

Figures 7 shows that 4DFG injected into mouse tail vein halts the growth in primary human GBM tumors in their flanks. After the first drug injection, all tumors were observed to shrink. The tumor volume remained static after the second and third injections, and tumor growth was only observed 48 hours after the last injection.
The upregulation of sugar transporters in glioma and in other cancers is an inviting targeting system utilizing differential drug uptake by cancer cells.

The two glucose/galactose transporters Glut3&14 were found to be associated with patient outcome after examining the TCGA mRNA dataset.

Using specific antibodies and our tumor tissue microarray, we found the levels of these transporters at the protein level were highly upregulated and that levels correlated with tumor grade.

The glucose/galactose mimetic 4-deoxy-4-fluoro galactose had previously been identified as an inhibitor of two enzymes involved in glycan synthesis, GALE and UGDH.

We have demonstrated in vitro, using lectin binding studies, that 4DFG is able to disrupt normal glycan synthesis and that alterations in glycan synthesis alter the pattern of glycan binding within the nucleus.

The incubation of 4DFG greatly affects cell growth in culture. At 48 hours incubation time, cells incubated with 4DFG had altered morphology and greatly enlarged nuclei.

In a flank model of primary human GBM, we were able to arrest tumor growth following an intravenous injection of 4DFG, and this drug was well tolerated by the animals.
Summary

The TCGA database suggest that many genes in the galactose/glycan pathway are upregulated in the gliomal of patient's with poor survival.

We found that the enzymes coded by these mRNA transcripts were also upregulated in glioma.

The ability of transporters Glut3&14 to take up glucose/galactose analogues was exploited to introduce an antimetabolite, glycan chain synthesis inhibitor into gliomas.

4DFG caused both aberrant expression glycans and antimitototic activity in gliomas.

Using 4DFG were able to arrest the growth of GBM xenografts in mouse flanks.

We are currently synthesizing more potent derivatives of 4DFG to create metabolic toxins during their metabolism by cancer cells.