A Molecular Screening Approach to Identify and Characterize Inhibitors of Glioblastoma Stem Cells

Koppany Visnyei, Hideyuki Onodera, Robert Danoiseaux, Kuniyasu Saigusa, Syuzanna Petrosyan, David De Vries, Denise Ferran, Jonathan Saxe, Eduard H. Panosyan, Michael Masterman-Smith, Jack Mottahedeh, Kenneth A. Bradley, Jing Huang, Chiara Sabatti, Ichiro Nakano, and Harley I. Kornblum

Abstract

Glioblastoma (GBM) is among the most lethal of all cancers. GBM consist of a heterogeneous population of tumor cells among which a tumor-initiating and treatment-resistant subpopulation, here termed GBM stem cells, have been identified as primary therapeutic targets. Here, we describe a high-throughput small molecule screening approach that enables the identification and characterization of chemical compounds that are effective against GBM stem cells. The paradigm uses a tissue culture model to enrich for GBM stem cells derived from human GBM resections and combines a phenotype-based screen with gene target-specific screens for compound identification. We used 31,624 small molecules from 7 chemical libraries that we characterized and ranked based on their effect on a panel of GBM stem cell-enriched cultures and their effect on the expression of a module of genes whose expression negatively correlates with clinical outcome: MELK, ASPM, TOP2A, and FOXM1b. Of the 11 compounds meeting criteria for exerting differential effects across cell types used, 4 compounds showed selectivity by inhibiting multiple GBM stem cells-enriched cultures compared with nonenriched cultures: emetine, α-arachidonoyl dopamine, α-oleyldopamine (OLDA), and α-palmitoyl dopamine. ChemBridge compounds #5560509 and #5256360 inhibited the expression of the 4 mitotic module genes. OLDA, emetine, and compounds #5560509 and #5256360 were chosen for more detailed study and inhibited GBM stem cells in self-renewal assays in vitro and in a xenograft model in vivo. These studies show that our screening strategy provides potential candidates and a blueprint for lead compound identification in larger scale screens or screens involving other cancer types. Mol Cancer Ther; 10(10); 1818–28.

Introduction

Glioblastoma (GBM) is almost universally fatal and new avenues of treatment are desperately needed. Several different lines of evidence exist to suggest that there are subpopulations of cells within GBM that have different capacities to initiate tumors in xenograft models. These tumor-initiating cells have at least some of the characteristics of stem cells in that they are capable of self-renewal and can produce the multiple cellular phenotypes that are found within the original tumor (1–3). Here, we use the term GBM stem cell to denote these similarities, but not necessarily to imply cell of origin. These GBM stem cells have been shown in a number of different ways to be relatively resistant to radiation and chemotherapy (4, 5).

Multiple efforts have been undertaken to isolate GBM stem cells to study and better understand their biology, as well as to develop therapies that target them. However, success has been limited so far. In fact, there is an increasing body of evidence that several such subpopulations may exist within one tumor and that their isolation might require the use of multiple marker systems (6, 7).

Hence, in an effort to derive large numbers of GBM stem cells that can be used for high throughput drug screening, we focused on more simple and reliable methods that might require the use of multiple marker systems (6, 7). Hence, in an effort to derive large numbers of GBM stem cells that can be used for high throughput drug screening, we focused on more simple and reliable methods that might require the use of multiple marker systems (6, 7). Hence, in an effort to derive large numbers of GBM stem cells that can be used for high throughput drug screening, we focused on more simple and reliable methods that might require the use of multiple marker systems (6, 7). Hence, in an effort to derive large numbers of GBM stem cells that can be used for high throughput drug screening, we focused on more simple and reliable methods that might require the use of multiple marker systems (6, 7). Hence, in an effort to derive large numbers of GBM stem cells that can be used for high throughput drug screening, we focused on more simple and reliable methods that might require the use of multiple marker systems (6, 7). Hence, in an effort to derive large numbers of GBM stem cells that can be used for high throughput drug screening, we focused on more simple and reliable methods that might require the use of multiple marker systems (6, 7).
proliferation or killed GBM stem cells derived from one tumor. We then further analyzed and prioritized candidate compounds that had selective effects on GBM stem cells from some tumors compared with those derived from others, or had selective effects on cultures enriched for GBM stem cells compared with those depleted of GBM stem cells. In a parallel approach, we also analyzed candidate compounds from the primary screen for their ability to inhibit the expression of genes that are associated with patient outcome (8). Through the use of this strategy, we have identified novel classes of compounds for the study of therapeutic approaches for attacking GBM stem cells. This study not only provides interesting candidates for further investigation but also represents a proof-of-principle for a screening paradigm that can be potentially used in a much larger scale for lead compound identification to develop new GBM stem cell-specific therapies.

Materials and Methods

Cell culture

Brain tumor specimens were collected following surgical resection at UCLA, with approval of Institutional Review Boards. Tumors were graded using World Health Organization guidelines. Samples were dissociated as previously described (1) in either neurosphere media or serum containing media. Neurosphere media contained DMEM/F12 supplemented with B27 (Invitrogen), bFGF (20 ng/mL, R&D Systems Inc.), epidermal growth factor (EGF; 50 ng/mL, Peprotech), penicillin/streptomycin (1%, Invitrogen), l-Glutamine (Invitrogen), and heparin (5 μg/mL, Sigma-Aldrich). Heparin, bFGF, and EGF were added to the media every 3 days. Spheres were passaged every 7 to 14 days following either dissociation with TrypLE Express (Invitrogen) or chopping using a tissue chopper (Geneq Inc.). Serum media contained DMEM/F12, 10% FBS and 1% penicillin/streptomycin. Under this condition, cells grew as attached monolayer cultures and were passaged when subconfluent. 293T, NHA, and human fetal astrocytes (gestational week 19) were cultured and expanded in serum-based media. Laminin-based adherent culture techniques were done following protocols described by Pollard and colleagues (ref. 9; see Supplementary Methods).

High-throughput molecule screen

The high-throughput screen (HTS) was done in a 384-well plate format using the ChemBridge DiverSet library (30,000 molecules; www.chembridge.com), and other collections of known bioactive compounds: Bioactive lipids, endocannabinoids, ion channel ligands, kinase and phosphatase inhibitors, orphan receptor ligands (204, 60, 72, 84, and 84 compounds, respectively; from www.enzolifesciences.com) and the Prestwick library [1120 Food and Drug Administration (FDA)-approved compounds; www.prestwickchemical.com], at concentrations recommended by the manufacturers. Cell number was estimated using ATPLite (PerkinElmer), and values were analyzed and corrected for systematic effects using a parametric model developed specifically for this screen (10). The Z’ factors in the 3 assays were 0.479, 0.53, and 0.51, respectively, indicating an assay system of good quality. All screen data were stored and managed online, on the Collaborative Drug Discovery platform (www.collaborativedrug.com). Chemical classifications and predicted biological functions of hit candidates were determined using online databases (Pubchem, Pubmed, and Lasso) and ADME software. Specific protocol details and hit candidate selection criteria are described in Supplementary Data.

Quantitative reverse transcriptase-PCR screen

For the quantitative reverse transcriptase (qRT)-PCR screen we used GBM146 that was cultured in sphere media. Spheres were dissociated and seeded into 96-well plates at 5 × 10^4 cells per well in sphere media. All experiments were done in duplicate. After 24 hours, compounds were added at 10 μmol/L and plates incubated for 6 hours at 37°C. Plates were then centrifuged, media removed and RNA extracted using the TRIzol method. RNA was transcribed into cDNA and gene expression levels quantified by RT-PCR using the PlatinumOnePlus system with SYBR Green method (Applied Biosystems). Expression levels of the target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase, plotted on a Log2 scale and Z-scores were calculated. Hit criteria were set at Z-score 1.50 or more. Expression levels of hit candidates were verified with qRT-PCR using larger cell numbers of different samples.

Clonal self-renewal assays and cell proliferation assays

To assess self-renewal capacity, cells were treated with compound or dimethyl sulfoxide (DMSO) and dissociated into a single-cell suspension. Equal numbers of live cells were seeded into 96-well plates, in fresh media, at clonal density, which was predetermined for each individual tumor culture by conducting mixing experiments using fluorescently labeled cells (11). These densities ranged between 5 and 10 cells per 96-well plate, depending on the GBM sample used. Three hours after plating, cell number was confirmed and plates were incubated until formation of spheres was observed. Spheres were fixed and incubated with Syto-9 dye (Molecular Probes) if not already expressing eGFP. Sphere number and size were assessed using an Acumen eX3 plate reader. For the low-passage sphere formation studies, GBM312 was used at passage 6. Proliferation studies were done using CFSE (carboxyfluorescein diacetate, succinimidyl ester) wash out and 0.51, indicating an assay system of good quality. All screen data were stored and managed online, on the Collaborative Drug Discovery platform (www.collaborativedrug.com). Chemical classifications and predicted biological functions of hit candidates were determined using online databases (Pubchem, Pubmed, and Lasso) and ADME software. Specific protocol details and hit candidate selection criteria are described in Supplementary Data.

Quantitative reverse transcriptase-PCR screen

For the quantitative reverse transcriptase (qRT)-PCR screen we used GBM146 that was cultured in sphere media. Spheres were dissociated and seeded into 96-well plates at 5 × 10^4 cells per well in sphere media. All experiments were done in duplicate. After 24 hours, compounds were added at 10 μmol/L and plates incubated for 6 hours at 37°C. Plates were then centrifuged, media removed and RNA extracted using the TRIzol method. RNA was transcribed into cDNA and gene expression levels quantified by RT-PCR using the PlatinumOnePlus system with SYBR Green method (Applied Biosystems). Expression levels of the target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase, plotted on a Log2 scale and Z-scores were calculated. Hit criteria were set at Z-score 1.50 or more. Expression levels of hit candidates were verified with qRT-PCR using larger cell numbers of different samples.

Clonal self-renewal assays and cell proliferation assays

To assess self-renewal capacity, cells were treated with compound or dimethyl sulfoxide (DMSO) and dissociated into a single-cell suspension. Equal numbers of live cells were seeded into 96-well plates, in fresh media, at clonal density, which was predetermined for each individual tumor culture by conducting mixing experiments using fluorescently labeled cells (11). These densities ranged between 5 and 10 cells per 96-well plate, depending on the GBM sample used. Three hours after plating, cell number was confirmed and plates were incubated until formation of spheres was observed. Spheres were fixed and incubated with Syto-9 dye (Molecular Probes) if not already expressing eGFP. Sphere number and size were assessed using an Acumen eX3 plate reader. For the low-passage sphere formation studies, GBM312 was used at passage 6. Proliferation studies were done using CFSE (carboxyfluorescein diacetate, succinimidyl ester) wash out and 0.51, indicating an assay system of good quality. All screen data were stored and managed online, on the Collaborative Drug Discovery platform (www.collaborativedrug.com). Chemical classifications and predicted biological functions of hit candidates were determined using online databases (Pubchem, Pubmed, and Lasso) and ADME software. Specific protocol details and hit candidate selection criteria are described in Supplementary Data.

Xenograft studies

Animal experimentation was done with institutional approval following NIH guidelines. To assess in vivo
tumor formation and growth, a dissociated cell suspension was stereotactically injected into the neostriatum of NOD-Scid gamma(null) mice. Animals were sacrificed when symptomatic or after 8 months if no symptoms developed, perfusion-fixed and the brain tissue sectioned on a cryostat. Tumor formation was determined based on immunohistochemical studies (see Supplementary Methods and Supplementary Fig. S1). For the drug treatment studies, cells stably expressing eGFP were used (12). Cells were exposed to either experimental drugs or DMSO ex vivo. After the incubation period, the drug was washed out and 50,000 live cells were transplanted in 2 μL of DMEM/F12. For the limiting dilution experiments, 500, 5,000, or 50,000 cells per animal were injected and mice were sacrificed 16, 12, or 10 weeks after transplantation, respectively. Tumor volumes were determined using fluorescence-based imaging and data analysis. For further details, see Supplementary Data.

Results

Human GBM cultured in EGF and FGF supplemented serum-free media are enriched in tumor-initiating GBM stem cells

Previous research has shown that patient-derived GBM samples propagated in bFGF and EGF supplemented serum-free media maintain their tumorigenic potential, while the same GBM samples lose their tumorigenicity if propagated in traditional serum-supplemented media (4). We cultured and orthotopically transplanted 9 GBM samples derived from different patients that we cultured under these 2 conditions (sphere media and serum media). Two to 3 months later, we observed tumor formation in all (41/41) animals that were injected with cells propagated in sphere media, whereas none of the 25 mice injected with serum-derived cells developed tumors (Supplementary Table S1). Hence, this culture model provides a suitable platform to investigate GBM stem cells and their non-tumorigenic counterparts in the frame of experiments that require large numbers of cells, such as HTS.

Screening of 30,000 small molecules revealed 694 compounds that negatively affected the proliferation and/or survival of GBM stem cell-enriched GBM cultures

We tested the effect of 30,000 small molecules derived from the ChemBridge library on one of our human GBM samples (GBM#107) that we cultured in sphere media to enrich for GBM stem cells. This sample was especially suitable for HTS because of its high proliferative nature and for its preference to grow as an adherent monolayer even in sphere media. The screen identified 694 compounds that significantly reduced cellular ATP concentration, as compared with DMSO-treated control wells, suggesting a negative effect on GBM stem cell proliferation, metabolism, and/or survival. These 694 compounds were further evaluated in subsequent screens.

A secondary screen identified 168 candidates with cell type-selective inhibitory effect

For the primary screen, we did not use a control cell line due to the large scale of the experiment. Therefore, we rescreened the 694 active compounds from the primary screen in a secondary screen on 2 GBM cell types (GBM107 and GBM1600) and on 293T human fibroblasts as a non-tumor control line. We then excluded all compounds that had an inhibitory effect on 293T cells only, and compounds inhibiting all 3 cell types, as nonspecific cytotoxins.

In addition to the 694 compounds identified in the primary screen, in this secondary round we also included multiple smaller compound collections, totaling 1,624 known bioactive compounds. By excluding nonspecific killers from this pool of 2,318 compounds, we identified 168 GBM stem cell-effective candidates (Supplementary Table S2). This compound collection was small enough to be further characterized using low-throughput strategies. For this, we pursued 2 different screening approaches as illustrated by the work flow chart in Fig. 1.

Eight compounds preferentially inhibited GBM stem cell-enriched GBM, compared with their non-GBM stem cell–enriched counterparts

To identify more selective compounds among our candidates, we screened them on an extended panel of different cell types and looked specifically for compounds that exhibited a differential effect profile. For this, we used several GBM samples (#107, 146, 157, 167, 217, and 1600) that were cultured either in sphere or serum media and the noncancer control cell types NHA (immortalized human astrocytes) and HFA (primary human fetal astrocytes). To control for effects of the different media, compound exposure all took place in sphere media, regardless of the media that cells were originally grown in. Although growth kinetics studies revealed only minor differences in proliferation rates between serum and sphere derived cells of the same cell type if cultured in screen (sphere) media, leaving little room for possible potentiating effects of the different media types, each screen condition was normalized to nondrug exposed controls of the same cell type (Supplementary Fig. S2). Compounds were ranked based on their differential effects among cell types. The exclusion/inclusion criteria were calculated as described in Supplementary Methods and included differential effects between: (i) tumor and nontumor control cells, (ii) between distinct GBM samples, and (iii) between GBM stem cell-enriched and non-GBM stem cell–enriched cultures of the same tumor sample. Because the HTS was done using a single drug concentration, we generated concentration–effect curves and calculated IC50 values for the top 30 compounds.

We identified 11 compounds [emetine, N-oleoyl dopamine (OLDA), N-palmitoyl dopamine (PALDA), N-arachidonoyl dopamine (NADA), anisomycin, campothecin, chrysenequinone, and the ChemBridge compounds #5485415, #5181524, #5211950, and #5560509]
that exhibited greater than one log(10) IC$_{50}$ difference between different cell groups described in criteria 1 to 3 above. Eight of these compounds exhibited a more than 10-fold lower IC$_{50}$ concentration in sphere cultures compared with serum cultures of at least one GBM sample, suggesting some selectivity in action against GBM stem cells (Table 1). In a separate experimental series using GBM cells that were cultured in serum and sphere media for different amount of times, we found that the differential compound effects between GBM stem cell-enriched and GBM stem cell-depleted cells were not due to a general protective effect of the serum-based media itself (Supplementary Data and Supplementary Fig. S3).

Out of these 8 compounds, emetine, OLDA, PALDA, and NADA showed selectivity of sphere versus serum grown cells across 5 (emetine) and 3 GBM samples (OLDA, PALDA, and NADA). OLDA, PALDA, and NADA share not only common structural but also biological characteristics, including an affinity to cannabinoid (CB1 and CB2) and to vanilloid (TRPV) receptors. Although activation of such receptors has been associated with decreased glioma growth (13–17), our studies using agonists and antagonists of these receptors suggest but do not absolutely prove that OLDA, PALDA, and NADA mediate their antitumor effect via other mechanisms (Supplementary Fig. 4A–D). From these compounds, we chose OLDA and emetine for subsequent characterization.

qRT-PCR screens revealed compounds that inhibit the expression of key GBM genes

Next, we wanted to find out whether we could identify compounds that had the ability to influence the expression of important GBM genes. GBMs express modules of genes whose expression varies with each other. Among these genes, some are called hubs in that their expressions are among the most highly correlated with those of other genes. In one module, identified previously as the mitotic module, the expression levels of these hub genes are inversely associated with patient outcome (8). We reasoned that regardless of mechanism, important regulators of GBM stem cell proliferation could ultimately lead to the downregulation of these key hub genes. Using an qRT-PCR approach, we quantified the effect of the 168 compounds derived from the secondary screen, on the expression of 4 hub genes, ASPM, MELK, FOXM1b, and TOP2A. We found 6 compounds that decreased the expression of MELK, 6 compounds that decreased ASPM, 7 compounds that
decreased TOP2A and 10 compounds that decreased FOXM1b. Two small molecule compounds from the ChemBridge library, #5560509 and #5256360 inhibited the expression of all 4 genes, while camptothecin, a topoisomerase-I inhibitor, and compounds #5402594, #5551547, #5349968, and #5256272 reduced the expression of 2 of the 4 genes simultaneously (Table 2).

To confirm the selective effect of the hit compounds on GBM stem cells, we conducted clonal sphere formation assays (18). Compound exposure significantly reduced the number of spheres formed, as compared with

Table 1. Screen identifies 8 compounds with differential effects on GBM stem cell-enriched and non-enriched GBM cultures

<table>
<thead>
<tr>
<th>Tested cell types</th>
<th>Emetine</th>
<th>OLDA</th>
<th>PALDA</th>
<th>NADA</th>
<th>Anisomycin</th>
<th>Camptothecine</th>
<th>Chrysenequinone</th>
<th>5485415</th>
</tr>
</thead>
<tbody>
<tr>
<td>107sph</td>
<td>0.2</td>
<td>0.7</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
<td>0.2</td>
<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>107ser</td>
<td>1.9</td>
<td>8.6</td>
<td>11.9</td>
<td>10.3</td>
<td>9.5</td>
<td>0.01</td>
<td>11.3</td>
<td>6.9</td>
</tr>
<tr>
<td>146sph</td>
<td>2.6</td>
<td>1.8</td>
<td>1.8</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146ser</td>
<td>48.6</td>
<td>36.0</td>
<td>39.0</td>
<td>37.7</td>
<td></td>
<td></td>
<td></td>
<td>5.3</td>
</tr>
<tr>
<td>157sph</td>
<td>0.3</td>
<td>1.1</td>
<td>1.4</td>
<td>1.0</td>
<td>1.8</td>
<td>0.3</td>
<td>1.1</td>
<td>5.4</td>
</tr>
<tr>
<td>157ser</td>
<td>32.5</td>
<td>4.8</td>
<td>5.0</td>
<td>4.5</td>
<td></td>
<td></td>
<td></td>
<td>47.6</td>
</tr>
<tr>
<td>167sph</td>
<td>0.1</td>
<td>1.1</td>
<td>1.7</td>
<td>1.2</td>
<td>2.0</td>
<td>0.4</td>
<td>0.4</td>
<td>5.3</td>
</tr>
<tr>
<td>167ser</td>
<td>50.4</td>
<td>7.5</td>
<td>7.4</td>
<td>9.7</td>
<td>89.2</td>
<td>6.5</td>
<td>5.4</td>
<td>6.0</td>
</tr>
<tr>
<td>217sph</td>
<td>0.4</td>
<td>1.9</td>
<td>2.1</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
<td>9.7</td>
</tr>
<tr>
<td>217ser</td>
<td>31.2</td>
<td>19.6</td>
<td>39.1</td>
<td>31.4</td>
<td></td>
<td></td>
<td></td>
<td>32.7</td>
</tr>
<tr>
<td>1600sph</td>
<td>0.1</td>
<td>2.2</td>
<td>2.1</td>
<td>2.2</td>
<td>11.0</td>
<td>0.4</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>1600ser</td>
<td>1.5</td>
<td>4.5</td>
<td>4.0</td>
<td>6.4</td>
<td>2.7</td>
<td>5.5</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>293T</td>
<td>4.5</td>
<td>1.8</td>
<td>1.9</td>
<td>1.7</td>
<td>8.8</td>
<td>0.0</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>NFA</td>
<td>10.7</td>
<td>18.2</td>
<td>11.1</td>
<td>17.2</td>
<td>18.6</td>
<td></td>
<td></td>
<td>8.4</td>
</tr>
</tbody>
</table>

NOTE: IC50 values are shown in μmol/L.
Abbreviations: Sph, sphere cultures; ser, serum cultures.

Table 2. qRT-PCR–based screens reveal 18 compounds inhibiting the expression of MELK, ASPM, TOP2A, and FOXM1b

<table>
<thead>
<tr>
<th>Library</th>
<th>Compound ID</th>
<th>MELK</th>
<th>ASPM</th>
<th>TOP2A</th>
<th>FOXM1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChemBridge</td>
<td>#5560509</td>
<td>-2.1</td>
<td>-3.5</td>
<td>-3.2</td>
<td>-1.5</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5256360</td>
<td>-2.6</td>
<td>-1.4</td>
<td>-4.0</td>
<td>-2.0</td>
</tr>
<tr>
<td>Prestwick</td>
<td>Camptothecine</td>
<td>-2.3</td>
<td>-1.5</td>
<td>-0.2</td>
<td>-0.5</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5402594</td>
<td>-0.2</td>
<td>-1.8</td>
<td>-2.4</td>
<td>-0.5</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5551547</td>
<td>-0.8</td>
<td>-1.8</td>
<td>-2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5349968</td>
<td>-0.7</td>
<td>-0.2</td>
<td>-3.2</td>
<td>-4.3</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5358272</td>
<td>-0.5</td>
<td>-1.0</td>
<td>-2.1</td>
<td>-2.3</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5354001</td>
<td>-0.7</td>
<td>-1.1</td>
<td>-1.7</td>
<td>-2.6</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5194403</td>
<td>-0.1</td>
<td>-0.5</td>
<td>1.0</td>
<td>-3.6</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5212518</td>
<td>-0.1</td>
<td>-0.3</td>
<td>-1.2</td>
<td>-2.2</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5316908</td>
<td>-0.6</td>
<td>-0.7</td>
<td>-1.1</td>
<td>-1.6</td>
</tr>
<tr>
<td>BIOMOL</td>
<td>Ro 31-8220</td>
<td>-2.7</td>
<td>0.8</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>BIOMOL</td>
<td>C8 Ceramime</td>
<td>-1.9</td>
<td>0.2</td>
<td>-0.7</td>
<td>-0.4</td>
</tr>
<tr>
<td>BIOMOL</td>
<td>6-Formylindolo[3,2-B]Carbazole</td>
<td>-1.7</td>
<td>0.6</td>
<td>0.6</td>
<td>-1.0</td>
</tr>
<tr>
<td>Prestwick</td>
<td>Chelidonine monohydrate</td>
<td>-0.2</td>
<td>-1.9</td>
<td>-1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Prestwick</td>
<td>Methiazole</td>
<td>-1.1</td>
<td>-1.5</td>
<td>-0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5217497</td>
<td>1.7</td>
<td>1.5</td>
<td>0.1</td>
<td>-1.8</td>
</tr>
<tr>
<td>ChemBridge</td>
<td>#5241816</td>
<td>-0.3</td>
<td>-0.7</td>
<td>-1.4</td>
<td>-1.7</td>
</tr>
</tbody>
</table>

NOTE: Degree of gene expression downregulation is expressed as Z scores; Z < -1.5 were considered hits (bold).
control cells, in a compound concentration and exposure time dependent manner (Fig. 2A–C). These results suggest that these compounds preferentially depleted the self-renewing cell population while relatively sparing the nonsphere forming ones. This effect diminished, but could be still observed after serial passaging of the primary spheres, suggesting a partial recovery of the sphere forming population upon drug removal (data not shown). These compounds also reduced the size of the clonally formed spheres and the total cell mass, in a dose-dependent manner. Interestingly, OLDA seemed to reduce the sphere number more than the sphere size or total cell number, which suggests a more selective inhibition of the sphere forming cells (Supplementary Fig. S5).

To further characterize the inhibitory effect of these compounds on our glioma cultures, we carried out cell proliferation experiments using CFSE washout. Results revealed a dose-dependent inhibition of cell proliferation (Supplementary Fig. S6).

Compounds #5560509, #5256360, OLDA, and emetine inhibited tumor formation in immunosuppressed animals

Next, we used an *ex vivo* treatment strategy (19) to determine whether some of the highest priority compounds also had an effect on the ability of the cells to form tumors and grow *in vivo*. For this, we implanted GBM stem cell-enriched tumor cells that were pretreated with emetine, #5560509, #5256360, or OLDA into the brain of immunosuppressed mice. We found a significantly reduced tumor mass in the compound-treated groups, compared with the vehicle-treated transplants, with almost no tumor mass present if the cells were exposed to #5560509 or #5256360 (Fig. 3 and Table 3). In addition, limiting dilution experiments using very small cell numbers in the same experimental setting suggest that the decreased incidence of tumor formation in the treated group is associated with the specific loss of tumor-initiating cells in the GBM samples upon drug treatment (Supplementary Table S3).
Discussion

Here, we have developed a screening strategy that enables the identification and categorization of chemical compounds based on their effect on GBM stem cells. These cells are particularly highly resistant to radiation therapy (5). Although they do show some sensitivity to Temozolomide (20), resistance is clearly present or develops, as the vast majority of tumors recur, even with this treatment. One of our goals was to determine whether some compounds selectively act on GBM stem cells compared with less tumorigenic cells from the same tumor. This selectivity may allow for the delineation of pathways and processes that are highly important to these cells. Furthermore, by making sure that a drug candidate has the potential to attack GBM stem cells, one might ensure the highest chance of therapeutic success. However, it is also important to note that such selectivity is not a critical requirement for the development of therapies, and may not even be desirable. The GBM stem cells component represents only a portion of cells in the tumor that may be the most highly sensitive to treatment.

Table 3. Xenograft studies show decreased tumor volume after ex vivo compound treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell no.</th>
<th>Mice transplanted</th>
<th>Observation time</th>
<th>Tumor volume (% of CTR)</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (CTR)</td>
<td>50,000</td>
<td>12</td>
<td>8 wks</td>
<td>100</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>#5560509</td>
<td>50,000</td>
<td>4</td>
<td>8 wks</td>
<td>2</td>
<td>0.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>OLDA</td>
<td>50,000</td>
<td>4</td>
<td>8 wks</td>
<td>27</td>
<td>9.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>#5256360</td>
<td>50,000</td>
<td>4</td>
<td>8 wks</td>
<td>4</td>
<td>3.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Emetine</td>
<td>50,000</td>
<td>4</td>
<td>8 wks</td>
<td>16</td>
<td>4.7</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 3. OLDA, emetine, and small molecules #5560509 and #5256360 inhibit ex vivo tumor formation. Representative brain sections showing decreased tumor formation in immunosuppressed animals after orthotopic transplantation of GBM stem cell-enriched GBM157 cells that were pretreated with experimental compounds. Graph shows quantitative tumor volume analysis. eGFP-expressing tumor cells seem black on scanned histology sections. *, P < 0.05; **, P < 0.01. Bars indicate the SE of the mean.
tumorigenic at the time of assay. It is possible that other cells in the tumor have the ability to take on a greater self-renewal and tumorigenic capacity over time, and drugs that attack both the GBM stem cells and the non-GBM stem cells component will be needed.

For our assays, we adopted a cell culture model that enriches GBM stem cell content of primary human GBM samples as described previously (4) and here. The major advantage in using this culture model to enrich for GBM stem cells is the ability to produce a large number of cells, which is a prerequisite for HTS and which can be problematic using alternative enriching techniques like FACS. Several cell-sorting approaches have been described to enrich for the tumor-initiating subpopulation of GBM. These include the use of cell surface markers CD133, SSEA-1 (CD15), Hoechst dye exclusion, or cell autofluorescence (6, 21–24). However, it is unclear whether any of these approaches can be used reliably and routinely to enrich for GBM stem cells across all GBM subtypes. For example, both CD133-positive and -negative cells possess self-renewal and tumor-initiating potential (25–27). In fact, self-renewing tumor-initiating cells do not necessarily consist of a single subpopulation of GBM cells that uniformly express a single cell surface marker. Rather, there may be multiple stem cell populations expressing different markers (6, 7, 28, 29). Another important factor to be considered is the interpatient heterogeneity of GBM that is fueled by an extensive repertoire of mutation patterns in this patient population (30, 31) that could conceivably give rise to GBM stem cells possessing very different sets of markers. Such markers or marker systems, once established, will be of great value and necessary to further explore the effect of hit candidates on different glioma subpopulations. The cell culture system we are using here seems to enrich for GBM stem cells across most GBM samples, independently of their mutational status or molecular characteristics. Although the degree to which the neurosphere cultures are enriched for GBM stem cells is not exactly known, the simplicity and practicality of this method to quickly and reliably expand GBM stem cell populations makes it more useful than FACS sorting, at least for purposes where a 100% pure GBM stem cell population is not a requirement for a successful experiment.

Although the large number of cells needed for HTS and the above described challenges of GBM stem cell enrichment make the use of freshly dissociated tumor tissue impossible, the use of an in vitro enrichment model raises potential concerns, as the artificial environment can change characteristics of the primary tumor cells. Furthermore, any in vitro study ignores the importance of the in vivo niche, an important component to understanding GBM stem cell biology (32). However, despite these drawbacks, there are important features of GBM stem cells that are preserved in the sphere culture model. Multiple studies have revealed that the sphere culture environment preserves many of the fundamental characteristics of the parent tumor, including cell heterogeneity (1) and the ability to form heterogeneous tumors in animal models, recapitulating the parent tumor’s cell composition and the invasiveness of GBM (2, 4). These cells and the tumors that they form maintain the genotypic and phenotypic signature of the original parent tumor that they were derived from (4). In addition, the capacity of the original tumor cells to form neurospheres is by itself an indicator of the in vivo aggressiveness of the tumor (12, 33, 34). Taken together, these data support the notion that the neurosphere culture technique is a valid and useful model to investigate at least some characteristics of GBM stem cells.

As a potential alternative to sphere-derived cultures, Pollard and colleagues have recently developed a laminin-based adherent culture technique that was shown to be suitable for HTS purposes (9). In that study the authors examined the effects of a relatively small group of compounds with known mechanism of action. Although this selection had only marginal overlap with our database, some compounds (or sometimes compounds from the same chemical family) were found to be effective in both studies. These were doxorubicin, nifedipin, fluphenazine/perphenazine, fluorastatin/taflastatin, chlorothiazid/phenothiazid, and fluoxetine. Although these compounds provide independent validation for both screening approaches, there were also compounds that seemed to be not effective in one study while effective in the other and vice versa. More study will be needed to determine whether these differences are due to the different GBM samples used, or due to the different culturing and screening approaches.

We carried out experiments comparing the laminin-based and the sphere culture methods side by side, using our top compound candidates. We found that our compounds can be efficacious in laminin-based cultures as well. However, the data showed a slight right-shift in the concentration effect studies in laminin-based cultures. The reason for this mild difference is not entirely clear, but it might be due to the smaller cell surface area that is available for the compounds to act on in the adherent compared with the floating cell cultures or it might be due to a protecting effect of the laminin itself (Supplementary Fig. S7).

We have shown that tumor cells that were exposed to experimental compounds ex vivo produced significantly reduced sized xenografts after transplantation into immunosuppressed animals, showing that the compounds affected cells that contribute to tumor initiation and/or tumor growth, and do not simply inhibit factors that contribute to growth in vitro. In addition to these findings, limiting dilution experiments using very small cell numbers in the same experimental setting suggest a specific loss of tumor-initiating cells caused by compound treatment. We do not yet know whether direct in vivo administration of these compounds will be effective. Further characterization and possibly chemical modification of the lead compounds will be needed before
extensive studies *in vivo* using already established tumors in experimental animals.

Our study complements a prior study (35), that used a different approach by probing for inhibitors of normal murine neural stem cell proliferation with the hypothesis that, because of the close relationship between neural stem cells and GBM stem cells, such compounds would be potential therapeutic candidates. That study screened 1,267 compounds and identified small molecules that are known to affect neurotransmission. Our screen similarly identified some of these neural stem cell-inhibiting compounds to have at least some effect on human GBM stem cells, including the dopamine antagonist Eticlopride hydrochloride, the serotonin antagonist Metyergoline, and the glutamate receptor blocker Ifenprodil tartrate. However, our screen also identified GBM stem cell-inhibiting compounds with known neuromodulating effects, like OLDA, that had no effect on normal neural stem cells and hence were not identified as hits in the above mentioned study.

We chose to screen several compound libraries combining uncharacterized and characterized small molecules. Using uncharacterized compounds carries the possibility of identifying novel lead candidates, but the mechanisms of action of these compounds are unknown. In contrast, collections of already FDA-approved drugs, as contained in the Prestwick library, enable a faster transition of potential hit candidates to clinical application (so called drug repurposing) and can also supply a particular screen with positive or negative controls. For example, our screen identified camptothecin, a known topoisomerase inhibitor that had been previously tested as an anticancer agent (36). Moreover, using a panel of substances with known mechanisms of action, one might gain additional information about the particular cell type used for the screen, or may assess the role of certain chemical classes for particular cell types (35). It is important to point out however, that even when one knows potential mechanisms of candidate compounds based on other studies, this does not necessarily mean that actions on GBM stem cells are mediated via these mechanisms. For example, OLDA, PALDA, and NADA are agonists for cannabanoid and vanilloid receptors and cannabinoind receptor agonists have known inhibitory effects on GBM. However, we found no evidence that these mechanisms are responsible for the effects we see on GBM stem cells using these compounds. To understand and identify the bona fide molecular targets of these active compounds, additional studies will be necessary utilizing techniques, such as the recently developed DARTS (37).

In our multistep HTS strategy, differential effect profile played a key role in the identification of specific and nonspecific inhibitors. We were interested in compounds that have the capacity to selectively inhibit GBM stem cells compared with other cells in the tumor, but also in compounds that had differential effects on different GBM samples. These latter ones are in fact potentially interesting candidates, as such a differential effect profile indicates a specific mechanism involving pathways that are vital for one GBM sample but not for the other.

One approach that we used to stratify compounds was to assay effects on the expression of downstream regulators of GBM proliferation. Several approaches have been undertaken to identify a subset of such genes, playing a key role in GBM initiation, proliferation, therapy resistance, and recurrence (38, 39). Global gene expression analysis of clinical GBM samples identified a gene coexpression module, consisting of key mitosis hub genes (8). Hub genes are those whose expression are most correlated with other genes of the expression module. Among these genes, the expression level of ASPM, FOXMI, MELK, PRC1, PTTG1, and TOP2A negatively correlate with patient survival (www.probesetanalyzer.com). While not all of these gene candidates have been characterized in great detail, knockdown of MELK has a direct inhibitory effect on GBM stem cell-like cell proliferation and self-renewal (40). We reasoned that compounds with the capacity to diminish expression of multiple (or all) hub genes would be potential regulators of highly critical processes in GBM. We identified 2 previously uncharacterized compounds #5365090 and #5256360 that inhibit the expression of at least 3 of the 4 genes that we investigated here.

Our screen also identified a number of compounds that inhibited one or a few of these genes (Table 2), some with previously identified biological activities. Camptothecin, a known topoisomerase I inhibitor, whose analogue atopotecan and irinotecan have been used extensively as cytotoxic agents in cancer therapy, downregulated the expression of both MELK and ASPM, uncovering new potential mechanisms through which this compound mediates its tumor growth inhibiting effect. Ro 31-8220, also known as bisindolylmaleimide, downregulated MELK expression. This compound has been described to inhibit protein kinase C, MAPKAP-K18 and p70 S6 kinase and has been proposed as a therapeutic agent in glioma (41–44). C8 ceramine, a compound that has been shown to induce nitric oxide synthase and cell death in medulloblastoma cells (45, 46), has also downregulated MELK expression in our screen. Chelidonine, that we identified as a *ASPM* downregulating compound, has been shown to induce apoptosis (47) and senescence by decreasing hTERT expression in human hepatocellular carcinoma cells (48) while its derivative, Ukrain has been shown to induce apoptosis in glioblastoma cells (49).

Our strategy used different types of tertiary screens where each approach delivered several hit compounds. No compound, however, seemed to meet all selection criteria. While such compounds might exist in libraries other than used here, the data imply that our different approaches are selecting for distinct compound qualities and therefore using parallel screening strategies in general might capture a broader range of compounds...
delivering more useful hits than sequential screening designs.

One important question is how our screening strategy differs in terms of hit recognition, from more traditional screening techniques that use established, serum-derived cancer cell lines. Such cell lines have an advantage in that they are easily expandable and serum-based media are much cheaper than the growth-factor supplemented media used here. However, these lines are likely to be depleted of the very cells that we are interested in, GBM stem cells, and therefore screens using them could miss compounds that show selectivity against GBM stem cells compared with the general tumor population. Thus, compounds, such as OLDA, PALDA, and NADA, that have ICS	's against serum-derived cultures of 10 to 15 μmol/L, would not be detected as hits in most screens using these cells.

In summary, we developed a screening strategy that marries phenotype-based high-throughput techniques with specific target-based low-throughput approaches, to categorize and characterize large number of compounds based on their effect on GBM stem cells. Using this approach, we identified several known and previously uncharacterized small molecule compounds that were effective against GBM stem cells using in vitro and in vivo assays, making them potential lead candidates for further drug development. Furthermore, our strategy can be adopted for large-scale screens involving more extensive and more diverse libraries to identify lead compounds for pharmacologic therapy of glioblastoma and potentially, other cancer types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Jong Sang Lee at the UCLA Molecular Screening Shared Resource for his help with the screens, Dr. Michael Haykinson and Ric Grambo at the Biological Chemistry Imaging Facility at UCLA for their help with the Typhoon imaging, Dr. Michael E. Jung for his expert advice, Dr. Paul Mischel for the GBM1600 cell line, and Dr. Eric Wechsler for the human astrocyte cultures. We also thank Andre Gregorian and Jantzen Sperry for outstanding technical assistance. The NHA line was obtained from Dr. Russell Peer.

Grant Support

Dr. Miriam & Sheldon Adelson Medical Research Foundation, NINDS grant NS052563 and NCI grant CA124974. R. Damoiseaux and K.A. Bradley were supported by NIH awards AI67769 and CA016042.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 14, 2011; revised August 9, 2011; accepted August 11, 2011; published OnlineFirst August 22, 2011.

References


Molecular Cancer Therapeutics

A Molecular Screening Approach to Identify and Characterize Inhibitors of Glioblastoma Stem Cells

Koppany Visnyei, Hideyuki Onodera, Robert Damoiseaux, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0268

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2011/08/17/1535-7163.MCT-11-0268.DC1

Cited articles
This article cites 48 articles, 13 of which you can access for free at:
http://mct.aacrjournals.org/content/10/10/1818.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/10/10/1818.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.