Identification of novel small-molecule inhibitors of glioblastoma cell growth and invasion by high-throughput screening

Lulu Wang¹², Hong Zhao², Kemi Cui²,*, Linli Yao¹, Min Ren³, Aijun Hao¹, Patrick Smollen², Fang Nie², Guangxu Jin², Qian Liu¹,*, Stephen TC Wong²

¹ Key Laboratory of the Ministry of Education for Experimental Teratology, Department of Histology and Embryology, School of Medicine, Shandong University, Ji'nan, Shandong, China; ² Department of Systems Medicine and Bioengineering, The Methodist Hospital Research Institute, Weill Cornell Medical College, Houston, TX, USA; ³ Department of Traditional Chinese Medicine, Qilu hospital, Shandong University, Ji'nan, Shandong, China

Summary
Glioblastoma multiforme (GBM) is the most common and lethal type of primary brain tumor with a very poor prognosis. Current therapies for GBM remain palliative and advances made in decades have resulted in only a slight improvement in treatment outcome. Exploring new therapeutic agents for GBM treatment, therefore, is of prime importance. In the present study, we performed a high-throughput screening for GBM cell growth and invasion, with an attempt to identify novel potential anti-GBM agents. An annotated compound library (LOPAC1280) of 1,280 pharmacologically active compounds was screened and ten compounds were validated and identified as inhibitors of GBM cell growth and invasion. Four of them, i.e., 6-nitroso-1,2-benzopyrone, S-(p-azidophenacyl) glutathione, phenoxybenzamine hydrochloride, and SCH-28080 have not been implicated in GBM cell growth and invasion previously, suggesting that they may serve as novel potential therapeutic agents for GBM treatment. In conclusion, novel inhibitors of GBM cell growth and invasion were identified in the present study, which provides a basis for the development of therapies for GBM, and may shed light on the molecular mechanisms underlying GBM cell behavior.

Keywords: Glioblastoma, screening, annotated compound library, cell growth, cell invasion

1. Introduction
Glioblastoma multiforme (GBM) is the most common and most aggressive malignant brain tumor. Current therapeutic approaches for GBM are predominantly palliative, including maximal surgical resection, radiotherapy, and chemotherapy (1). However, one of the most important barriers to successful therapy is the diffuse invasion of GBM cells, making them elusive targets for effective surgical management and giving rise to tumor recurrence. Besides, lack of a significant antitumor effect from traditional therapies is frequently observed (2), resulting in quite low relative survival (five-year survival rates are less than 5%) (The Central Brain Tumor Registry of the United States, 2012). Therefore, identifying novel effective therapeutic agents for treating GBM is urgently needed.

Etiology and pathophysiology of GBM are complex and currently unclear. Potential molecular targets for GBM therapies are being explored while effective ones are still largely unknown. As GBM is characterized by high proliferation rate and diffuse invasion, regulators targeting these cell behaviors may lead to immediate clinical improvement. The development of experimental technologies to measure cell growth or invasion has made it possible to acquire potential anticancer compounds, while a major obstacle...
is that the current cell invasion assays are difficult to perform in parallel at the scale required to screen large libraries (3). High-throughput approaches, which allow simultaneous screening of hundreds to thousands of compounds, are therefore of interest to basic scientists and those in search of therapeutics (4). Bioluminescent assays, which are based on the detection of ATP by the luciferin-luciferase reaction, are being studied for high-throughput screening due to their sensitivity, broad linearity, and robustness to library compounds and complex biological samples (5). A bioluminescent-based assay for GBM cell growth and invasion was developed in our laboratory previously (6), and it was adapted here to allow the possibility of screening regulators of GBM cell behavior in a high-throughput manner.

In the present study, using a high-throughput screening approach, we screened an annotated compound library (LOPAC1280) of 1,280 pharmacologically active compounds that influence most cellular processes and cover all major drug target classes, to identify novel potential therapeutic agents against GBM. LOPAC1280 represents a collection of compounds from 56 pharmacological classes with well-characterized activities and experimentally validated biological mechanisms, and thus, this screening may also provide new insights into the molecular basis of GBM.

2. Materials and Methods

2.1. Cells, cell cultures and compounds

Human glioblastoma cell lines U87MG and U251MG were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS). U87-luc (U87MG glioblastoma cells genetically engineered to express firefly luciferase reporter gene) was kindly provided by Santosh Kesari (Dana-Farber Cancer Institute, Harvard Medical University) and was maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% l-glutamine, and 0.5 mg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in a humidified tissue culture incubator at 37°C with a 5% CO2 atmosphere.

Compounds from LOPAC1280 (Sigma-Aldrich, St. Louis, MO, USA) were received as 10 mM DMSO stock solutions and stored at −20°C until further use. For validation experiments, active compounds (i.e., "hits") from the primary high-throughput screening were prepared at a concentration ranging between 0.01 and 100 μM.

2.2. High-throughput screening of LOPAC1280

For primary screening, a high-throughput screening assay for cell growth and invasion was performed and a library of 1,280 compounds was screened. Briefly, the luciferin-immobilized 96-well black-wall clear-bottom plates (Becton Dickinson, Franklin Lakes, NJ, USA) were prepared following the procedure described in our previous paper (6) (Figure 1A). For each plate, the top half (rows A-D) was not coated with luciferin, with the aim of evaluating cell proliferation. Wells in rows E-H were coated with luciferin on the bottom to evaluate cell invasion. Each compound of the LOPAC1280 library was mixed with U87-Luc cell conditioned media and Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA, USA), which had been widely used in tumor cell invasion assays or functioned as 3D cell culture platforms (7,8), to a final concentration of 10 μM (the DMSO concentration was 0.1% for all samples and controls). Then the mixture was added onto the 96-well plates in triplicate in a cold room at 4°C. Each 96-well plate was placed in an incubator at 37°C for 2 h. Next, luciferin (1 μL) was placed in each of the wells in rows A-D. About 5,000 U87-Luc cells in 5 μL media were loaded onto the Matrigel (0 h) in all wells followed by incubation in a humidified incubator at 37°C in a 5% CO2 atmosphere for 24 h. The bioluminescent signals were detected at 0 h and 24 h using the IVIS 100 system (Xenogen Co., Alameda, CA, USA). Signal intensity was quantified using Living Image software (version 2.50.1, Xenogen Co., Alameda, CA, USA) (in terms of photon counts).

A good linear correlation between the U87-Luc cell number in Matrigel and bioluminescent signal had been obtained in our previous study (6). The photon counts of wells in rows A-D (no luciferin coating) reflected the total number of cells and those in rows E-H (luciferin coated) reflect the cells that moved through the Matrigel. Accordingly, cell growth and invasion were measured. For each 96-well plate, 15 compounds were evaluated. Z' factor was used for quality control of the screening plates, which was defined as described in the following equation: \[ Z' = \frac{3\sigma_s + 3\sigma_b}{|\mu_b - \mu_s|} \]
where \(\sigma_s\) represents the S.D. of signal (σs) or background (σb) and \(\mu_s\) and \(\mu_b\) is the mean and \(\mu\) represents the mean (9).

2.3. Cell growth assay

Cell growth was assessed by CellTiter 96® AQueous cell proliferation assay (MTS) (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, 10,000 U87MG or U251MG cells in 100 μL medium were seeded in triplicate in each well of the 96-well white-wall clear-bottom plates and incubated overnight, followed by 24 h treatment with active compounds at different concentrations (0.01 μM to 100 μM). Cells were then washed and fresh medium was added followed by Cell Titer 96® AQueous One solution (20 μL/well). After 3 h incubation, absorbance at 490 nm was measured using the FLUOstar Omega Microplate Reader (BMG LABTECH, Cary, NC, USA).
Figure 1. Results of LOPAC1280 high-throughput screening for anti-GBM compounds and chemical structures of the validated novel hits. (A) Layout of the 96-well plate for LOPAC1280 high-throughput screening. Wells in rows A-D have no luciferin coating. U87-Luc cells and luciferin were added simultaneously and cell growth was monitored. Bottom of wells in rows E-H were coated with luciferin. U87-Luc cells alone were added and the bioluminescent signals could be detected only if the cells invade the Matrigel and reached the bottom of wells. Columns 10-12, rows D and H, control cells treated with 0.1% DMSO; Other wells, cells treated with LOPAC1280 compounds (10 μM). Relative luciferase activity of the control was defined as 100% and measurements from other wells were calculated accordingly. For each 96-well plate, 15 compounds were evaluated for their activity in GBM cell growth and invasion. (B) Results: 1,280 compounds were screened in triplicate, leading to an initial list of 25 active compounds, of which ten were subsequently validated in traditional assays. The ten validated compounds and their effects on U87-Luc cell growth and invasion as evaluated by the high-throughput screening are shown in (C). Values in bar graphs represent mean ± S.D. of three independent experiments. * p < 0.05 vs. control. Four (i.e., 6-nitroso-1,2-benzopyrone, S-(p-azidophenacyl) glutathione, phenoxybenzamine hydrochloride, and SCH-28080) of the ten validated compounds were identified as novel inhibitors of GBM cell growth and invasion. Their chemical structures are shown in (D).
2.4. Cell invasion assay

Cell invasive capacity was evaluated by 24-well Matrigel™ Invasion Chamber according to the manufacturer’s instructions (BD Biosciences). Briefly, 25,000 U87MG or U251MG cells in 500 μL medium containing active compounds at different concentrations were added in triplicate to the inserts and incubated for 24 h. After the removal of non-invading cells with a cotton swab, cells on the lower surface of the membrane were stained with a Diff-Quik stain kit (Dade-Behring, Newark, DE, USA). Cells were counted in five chosen microscope fields in each well and the results were numerically averaged. Cell invasion was calculated according to the manufacturer’s manual.

2.5. Cell migration assay

Cell migration was examined by wound-healing assay as described previously (10). Relative migration distance was quantified by measuring the advance of the two opposing wound edges at 0 h and 12 h using Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

2.6. Statistical analysis

Data are presented as the mean ± S.D. Statistical significance was determined by one-way ANOVA, followed by Dunnett’s post-hoc test. All tests were 2-sided and a value of p < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism for Windows (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. High-throughput screening for novel inhibitors of GBM cell growth and invasion of LOPAC1280

As an initial step to discover novel potential compounds against GBM, 1,280 pharmacologically active compounds from 56 pharmacological classes were quantitatively assessed for their effects on GBM cell growth and invasion at a concentration of 10 μM (Figure 1A). Assay quality was evaluated using Z’-factors (see Materials and Methods). A Z’-factor of 1 is considered ideal; a Z’-factor between 0.5 and 1.0 represented excellent assays (9). For the screen plates, an average Z’ factor of 0.513 was obtained, indicating that the assay was acceptable for cell-based screening (11). Outlier wells caused by plate preparation mishandling were not included in the analysis.

The high-throughput screening of LOPAC1280 resulted in an initial identification of 25 inhibitors (Figure 1B). As will be shown below, ten of them were validated in traditional cell growth, invasion and migration assays. They belong to several pharmacological classes, suggesting that these classes should be paid more attention to in the development of therapies for GBM.

It is gratifying that carmustine, I-OMe-Tyrphostin AG 538, (−)-perillic acid, 13-cis-retinoic acid, vincristine sulfate, and Taxol, which had been studied in preclinical experiments, evaluated in clinical trials, or used in clinical as chemotherapeutic agents for tumors including GBM (see Discussion for details), were identified in our screening (Table 1). The other four, i.e., 6-nitroso-1,2-benzopyrone, S-(p-azidophenacyl) glutathione, phenoxybenzamine hydrochloride and SCH-28080, had not been implicated in GBM cell behaviors and were therefore identified as novel potential anti-GBM agents (Table 1, Figure 1D).

<table>
<thead>
<tr>
<th>LOPAC Cat. #</th>
<th>Name of compound</th>
<th>Pharmacological class</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 0400</td>
<td>Carmustine</td>
<td>DNA</td>
<td>DNA alkylating agent; causes interstrand crosslinks</td>
</tr>
<tr>
<td>T 7697</td>
<td>I-OMe-Tyrphostin AG 538</td>
<td>Phosphorylation</td>
<td>Insulin growth factor 1 (IGF-1) receptor protein tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>P 7083</td>
<td>(−)-Perillic acid</td>
<td>G protein</td>
<td>Interferes with activity of p21 ras and other small G proteins by inhibiting post-translational cysteine isoprenylation</td>
</tr>
<tr>
<td>R 3255</td>
<td>13-cis-Retinoic acid</td>
<td>Transcription</td>
<td>Anti-inflammatory and antitumor actions mediated through RAR-beta and RAR-alpha receptors</td>
</tr>
<tr>
<td>V 8879</td>
<td>Vincristine sulfate</td>
<td>Cytoskeleton and ECM</td>
<td>Inhibitor of microtubule assembly</td>
</tr>
<tr>
<td>T 7402</td>
<td>Taxol</td>
<td>Cytoskeleton and ECM</td>
<td>Antitumor agent; promotes assembly of microtubules and inhibits tubulin disassembly process</td>
</tr>
<tr>
<td>N 8403</td>
<td>6-Nitroso-1,2-benzopyrone</td>
<td>Transcription</td>
<td>Poly (ADP-ribose) polymerase (PARP) ligand which preferentially destabilizes one of the two zinc-fingers, inactivating the enzyme Glyoxalase and glutathione S-transferase inhibitor</td>
</tr>
<tr>
<td>A 1782</td>
<td>S-(p-Azidophenacyl) glutathione</td>
<td>Multi-drug resistance</td>
<td></td>
</tr>
<tr>
<td>B-019</td>
<td>Phenoxybenzamine hydrochloride</td>
<td>Adrenoceptor</td>
<td>Selective alpha adrenoceptor blocking agent; calmodulin antagonist</td>
</tr>
</tbody>
</table>
3.2. Effects of the active compounds on GBM cell growth

A traditional cell viability assay (MTS assay) was performed to achieve a dose-response evaluation of the active compounds in two GBM cell lines, U87MG and U251MG. The concentration used in the primary screening was 10 μM (see Materials and Methods). In this part, a concentration ranging between 0.01 and 100 μM was applied (Figure 2). All the four compounds caused a dose-dependent inhibition of GBM cell growth, although the degree of inhibition varied.

3.3. Effects of the active compounds on GBM cell invasion

The invasive potential of GBM cells was tested using a Matrigel cell invasion assay and effects of the four novel inhibitors are shown (Figure 3). Compared to the control, 1 μM 6-nitroso-1,2-benzopyrone, 1 μM S-(p-azidophenacyl) glutathione, 10 μM phenoxybenzamine hydrochloride and 10 μM SCH-28080 significantly reduced the invasion of U87MG cells to 79.8%, 65.2%, 48.6%, and 52.9%, respectively. A similar result was obtained in U251MG cells.

3.4. Effects of the active compounds on GBM cell migration

Additionally, we examined whether the reduced invasiveness of GBM cells caused by the hit compounds was accompanied by reduced cell motility. A cell migration assay (wound healing assay) was employed (Figure 4). As shown, compared to the control, 1 μM 6-nitroso-1,2-benzopyrone and 1 μM S-(p-azidophenacyl) glutathione significantly reduced U87MG cell migration to 86% and 80%, respectively. When treated with 10 μM phenoxybenzamine hydrochloride or 10 μM SCH-28080, U87MG cell migration was reduced to 72% or 82%, respectively. The decreases in U251MG cell migration were also

Figure 2. Dose-response curves displaying the activity of the four active compounds in GBM cells. Two GBM cell lines (U87MG and U251MG) were treated with various concentrations of 6-nitroso-1,2-benzopyrone (A) S-(p-azidophenacyl) glutathione (B) phenoxybenzamine hydrochloride (C) and SCH-28080 (D) for 24 h. Cell growth was measured by MTS cell viability assay. Cell viability was calculated as the ratio of the corrected absorbance value of the treated cells to that of untreated control cells. The results are presented as mean ± S.D. of three independent experiments.
significant. In other words, as expected, all four compounds caused a statistically significant reduction in the cell migration.

Parallel experiments for evaluating effects of different concentrations of DMSO (used in this study) on GBM cell proliferation, invasion, and migration were performed to rule out the possibility that DMSO might have subtle effects on GBM cell behaviors. No significant difference was observed after DMSO treatment in each experiment (data not shown).

4. Discussion

Mechanisms underlying GBM cell proliferation, invasion, and migration are very complicated. Regulators of these biological behaviors, which hold the promise of effective therapeutic approaches for treating GBM, still have not been well defined. In this study, all compounds of the LOPAC1280, which is a versatile library including the latest drug-like molecules in the fields of cell signaling and neuroscience, and reflects the most commonly screened targets in the drug discovery community, were evaluated in the primary screening assay. The four newly identified potential anti-GBM compounds come from distinct pharmacological classes, some of which have never been implicated in tumor cell behaviors suggesting that these classes may be involved in diverse biological processes of GBM. Other unrevealed compounds from

---

**Figure 3. Effects of the four active compounds on GBM cell invasion.** (A) U87MG and U251MG cells were treated with the active compounds from the primary high-throughput screening for 24 h. Cell invasion was evaluated by Matrigel cell invasion assay. The invaded cells that adhered to the lower surface of the membrane were counted. The invasive potential of the treated cells is presented as a percentage invasion of control. Data represent mean ± S.D. of three independent experiments. *p < 0.05 vs. control. (B) The representative images of U87MG cells are shown. Scale bar, 50 μm.

**Figure 4. Effects of the four active compounds on GBM cell migration.** (A) U87MG and U251MG cells were treated with the active compounds from the primary high-throughput screening for 12 h, and cell migration was measured by wound-healing assay. Cell migration is presented as percentage of relative migration distance divided by that of the control. Each value represents the mean ± S.D. of three independent experiments. *p < 0.05 vs. control. (B) The representative images of U87MG cells are shown. Scale bar, 100 μm.
these classes may also be worth noting in terms of their anticancer effects.

In this study, ten validated active compounds were obtained, of which six have been previously reported to be implicated in GBM cell behaviors (Table 1). Carmustine is a DNA alkylating agent and causes interstrand crosslinks, and it has long been used as a treatment for GBM (12). I-OMe-Typhostin AG 538 is an insulin growth factor 1 (IGF-1) receptor (IGF-1R) protein tyrosine kinase inhibitor. IGF-1 has been well discussed in GBM (13), and targeting IGF-1R has been studied as a treatment option for GBM (14). (--)-Perillic acid is a metabolite of perillyl alcohol (POH), which has shown a therapeutic anticancer effect and is currently under phase I and II clinical trials for human cancers including GBM (15,16). 13-cis-Retinoic acid (cRA) has antitumor actions mediated through RAR-beta and RAR-alpha receptors, which has long been evaluated and has shown therapeutic effectiveness in clinical trials in treatment of brain tumors (17). Vincristine sulfate and Taxol, both from the class of cytoskeleton and ECM, are being clinically evaluated alone or combined for use against gliomas (18,19). The identification of these compounds is gratifying and may help validate this screen as an effective means to identify compounds that regulate GBM cell behavior. Moreover, the therapeutic effects of these compounds in clinical studies suggest that the four newly identified compounds in our study (i.e. 6-nitroso-1,2-benzopyrone, S-(p-azidophenacyl) glutathione, phenoxybenzamine hydrochloride, and SCH-28080), might benefit patients with GBM and are therefore worth further study (Table 1).

6-Nitroso-1,2-benzopyrone is a poly(ADP-ribose) polymerase (PARP) ligand that has been reported to suppress proliferation of leukemia and other malignant human cells (20), while few studies have focused on its effect on GBM cell behavior.

S-(p-Azidophenacyl) glutathione is a glyoxalase and glutathione S-transferase (GST) inhibitor. It has been reported that blockage of glyoxalase resulted in an inhibition of proliferation of human leukemia cells (21). GSTs have been implicated in the development of drug resistance (22), and may be responsible for poor response to alkylating agents in GBM treatment (23). In this study, the effects of S-(p-azidophenacyl) glutathione on GBM cell behavior were examined for the first time.

Phenoxybenzamine hydrochloride is a selective alpha adrenoceptor blocking agent as well as a calmodulin antagonist. The alpha adrenoceptor antagonist has been extensively studied in human prostate cancer and shown to be involved in cell cycle arrest and apoptosis (24), whereas it has rarely been studied in GBM cells. Based on the distribution of alpha-adrenoceptors in the human central nervous system, our findings are of concern (25). Besides, phenoxybenzamine hydrochloride also reacts irreversibly with calmodulin (26), which has been shown to play roles in astrocytoma cell growth (27). In this connection, our finding of the effects of phenoxybenzamine hydrochloride in GBM cell behavior is attractive.

SCH-28080 is a potent inhibitor of gastric H⁺-and K⁺-ATPase, which was considered to exert its antisercretory effect by a competitive interaction with the high affinity K⁺-site of the gastric ATPase (28). It has been shown to inhibit basal and stimulated aminopyrine accumulation in isolated gastric glands, and to cause relaxation of human airway smooth muscle (29,30). Here SCH-28080 exhibited antitumor activity against GBM in vitro, whereas the underlying molecular mechanisms still need to be elucidated.

The four active compounds, which have not been previously implicated in GBM cell behavior, are all shown in our study to exert inhibitory effects on GBM cell growth and invasion. This favorable result allows them to be further studied as novel potential therapeutic agents against GBM.

It should be mentioned that although these compounds showed favorable effects on GBM cells in vitro, there is still a long way to go before they benefit patients. Their pharmacokinetic and pharmacodynamic activities need to be addressed, and their effectiveness crossing the blood-brain barrier and delivery to a brain tumor area are of vital concern (31). Good news is that scientists have never stopped devising innovative approaches to circumvent these obstacles, and strategies for increasing drug delivery are being discovered (32). These agents also need further investigation for their cancer-specific action because the toxicity of anticancer agents against normal cells is still one of the major challenges in cancer treatment (33). While these uncertainties could not stop this study from providing starting points for anti-GBM drug discovery and design.

In summary, via a high-throughput screening, from an annotated compound library, we identified four novel small-molecule inhibitors of GBM cell growth and invasion, with an attempt to provide useful therapeutic leads for GBM treatment. Currently, these four active compounds are being investigated in our laboratory to explore the underlying mechanisms, and their anti-tumor effects are also being evaluated in an intracranial GBM xenograft model. The results will be reported in detail elsewhere.

Acknowledgements

The work was supported by NIH/NCI, U54 CA149196 and Bioinformatics and Bioengineering Program at The Methodist Hospital Research Institute (TMHRI, SWONG) of US. Natural Science Foundation of Shandong University (No. 2012JC006).
References


www.biosciencetrends.com


(Received June 15, 2012; Revised August 3, 2012; Accepted August 7, 2012)