Pyriminium Targets CD133 in Human Glioblastoma Brain Tumor-Initiating Cells

Chitra Venugopal1, Robin Hallett2, Parvez Vora1, Branavan Manoranjan1,3,4, Sujeivan Mahendram1, Maleeha A. Qazi1,4, Nicole McFarlane1, Minomi Subapanditha1, Sara M. Nolte1, Mohini Singh1,4, David Bakhshiyani1,4, Neha Garg1, Thysanthy Vijayakumar1, Boleslaw Lach5, John P. Provias5, Kesava Reddy6, Naresh K. Murty6, Bradley W. Doble1,4, Mickie Bhatia1,4, John A. Hassell2,4, and Sheila K. Singh1,4,6

Abstract

Purpose: Clonal evolution of cancer may be regulated by determinants of stemness, specifically self-renewal, and current therapies have not considered how genetic perturbations or properties of stemness affect such functional processes. Glioblastoma-initiating cells (GICs), identified by expression of the cell surface marker CD133, are shown to be chemoresistant. In the current study, we sought to elucidate the functional role of CD133 in self-renewal and identify compounds that can specifically target this CD133+ treatment-refractory population.

Experimental Design: Using gain/loss-of-function studies for CD133 we assessed the in vitro self-renewal and in vivo tumor formation capabilities of patient-derived glioblastoma cells. We generated a CD133 signature combined with an in silico screen to find compounds that target GICs. Self-renewal and proliferation assays on CD133-sorted samples were performed to identify the preferential action of hit compounds. In vivo efficacy of the lead compound pyriminium was assessed in intracranial GIC xenografts and survival studies. Lastly, microarray analysis was performed on pyriminium-treated GICs to discover core signaling events involved.

Results: We discovered pyriminium, a small-molecule inhibitor of GIC self-renewal in vitro and in vivo, in part through inhibition of Wnt/β-catenin signaling and other essential stem cell regulatory pathways. We provide a therapeutically tractable strategy to target self-renewing, chemoresistant, and functionally important CD133+ stem cells that drive glioblastoma relapse and mortality.

Conclusions: Our study provides an integrated approach for the eradication of clonal populations responsible for cancer progression, and may apply to other aggressive and heterogeneous cancers. Clin Cancer Res; 21(23); 5324–37. ©2015 AACR.

Introduction

Despite improvements in cancer treatment, many patients experience disease progression, relapse, and reduced overall survival. Prior research has focused on molecular mechanisms or genetic alterations implicated in drug resistance. However, the contribution of intratumoral heterogeneity to therapy failure and relapse must be acknowledged (1). Individual tumor cells can display variable proliferation, apoptosis, metabolism, and other “hallmarks of cancer” (2). Therefore, mechanisms driving intratumoral cellular variability present appealing therapeutic targets.

Intratumoral cancer cell heterogeneity was identified by prospective isolation via marker-directed cell sorting of malignant stem-like cell subpopulations, with corresponding phenotypic diversity. The repertoires of cell surface markers that identify tumor-initiating cells (TICs) across multiple solid tumors include CD133 (3–6), Stem cell antigen 1 (Sca1; refs. 7–10), CD44 (11, 12), CD24 (13, 14), and epithelial-specific antigen (ESA; refs. 13, 15). However, unlike clinical biomarkers (like EGFR, HER2, and KRAS), the functional significance of these proteins in tumor progression remains poorly understood. Different cell surface markers may indicate heterogeneous TIC subpopulations with different stem cell characteristics, degrees of differentiation, and malignant biologic behaviors. Moreover, using surface markers to dissect intratumoral heterogeneity is complicated by cancer cells alternating between TIC and non-TIC states (16). Determinants of stemness have been shown to contribute to treatment failure, irrespective of whether the tumor cell population exists in dynamic equilibrium. Self-renewal, the cardinal property of stemness, is defined by the ability of a cell, at each cell division, to generate an identical copy of itself and a cell of the same or different phenotype (17). Cancer may thus be thought of as a disease of unregulated self-renewal (18).

CD133 is a marker of self-renewing hematopoietic (19) and neural (20) stem cells that also identifies TIC populations in multiple human cancers (3–6). CD133 expression correlates with
Pyrvinium Targets CD133 in Human Glioblastoma

**Translational Relevance**

Glioblastoma (GBM) is one of many highly aggressive, heterogeneous, and treatment-refractory human cancers, with no truly efficacious treatment option, and patients inevitably relapse following current standard chemoradiotherapy. A distinct pool of cancer stem-like cells or glioblastoma-initiating cells (GICs) within human GBM impart tumorigenicity and resistance to conventional therapy. Numerous studies have implicated CD133⁺ GICs as drivers of chemoresistance and radioresistance. We have designed a novel in silico, in vitro, and in vivo drug discovery approach to target treatment-refractory CD133⁺ GBM cells that evade current therapy. We discovered that pyrvinium, an FDA-approved antihelminthic compound, reduces in vitro and in vivo self-renewal and tumor-initiating capability through targeting of CD133⁺ GICs. This novel drug discovery approach can be applied to other prospectively identified GIC populations that drive tumor heterogeneity, to allow for strategic targeting of multiple functionally relevant stem cell populations that may be implicated in tumor recurrence and patient relapse.

Disease progression, metastasis, recurrence, and poor overall survival in several human malignancies (21, 22), but insight into its function remains limited (23, 24). Although CD133 is the first identified member of the Prominin family of pentaspan membrane glycoproteins, with an implied signaling role, the functional significance of CD133 in modulating intratumoral heterogeneity via self-renewal regulation is unclear.

Here, we describe an integrative approach for the treatment of human glioblastomas (GBM), aggressive brain tumors that remain incurable despite surgical excision and chemoradiotherapy. Therapeutic failure is in part due to tumor cell heterogeneity that imparts phenotypic diversity (25, 26). Current knowledge of glioblastoma-initiating cells (GICs) is based on comparative studies of bulk tumors, enriched or depleted for GIC markers such as CD133 (6, 27) or CD15 (28), which may not capture the dynamic nature of GICs (29, 30). We describe a strategic drug discovery platform to identify compounds that inhibit self-renewal, offering a means to selectively eradicate evolving cellular subpopulations that drive tumor initiation, maintenance, and relapse.

**Materials and Methods**

**Dissociation and culture of primary GBM tissue**

Human GBM samples (Supplementary Tables S1 and S6) were obtained from consenting patients, as approved by the Hamilton Health Sciences/McMaster Health Sciences Research Ethics Board. Samples were dissociated in artificial cerebrospinal fluid containing 0.2 Wünsch unit/mL Liberase Blendzyme 3 (Roche), and incubated at 37°C in a shaker for 15 minutes. The dissociated tissue was filtered through a 70-μm cell strainer and collected by centrifugation (1,500 rpm, 3 minutes). Tumor cells were resuspended in a serum-free tumor stem cell medium (TSM), and plated on ultra-low attachment plates (Coming). Our complete TSM per 500 mL includes: Dulbecco’s modified Eagle’s medium/F12 (480 mL; Invitrogen), N2-supplement (5 mL; Invitrogen), 1 mol/L HEPES (5 mL; Wisent), glucose (3 g; Invitrogen), N-acetylcysteine (1 mL of 60 mg/mL solution; Sigma), and neural survival factor-1 (10 mL; Lonza). Added growth factors include human recombinant epidermal growth factor (20 ng/mL; Invitrogen), basic fibroblast growth factor (20 ng/mL Invitrogen), leukemia-inhibitory factor (10 ng/mL; Chemicon), and antimycotic (10 ng/mL; Wisent). Red blood cells were lysed using ammonium chloride solution (STEMCELL Technologies).

**Propagation of GICs**

Neurospheres derived from minimally cultured human GBM samples were propagated as previously described (31). Adherent cells were replated in low-binding plates and cultured as tumorspheres, which were maintained as spheres upon serial passaging in vitro. These cells retained their self-renewal potential and were capable of multilineage differentiation. Cell lines were subtyped based on the expression of 21 subtype-specific genes, as described by Verhaak and colleagues (32). The GBM sample subtypes are listed in Supplementary Table S6.

**Secondary sphere formation assay**

Tumorspheres were dissociated using 5 to 10 μL Liberase Blendzyme 3 in 1 mL PBS for 5 minutes at 37°C. Cells were plated at 200 cells per well in 200 μL of TSM in a 96-well plate. Cultures were left undisturbed at 37°C with 5% CO₂. After 7 days, the number of secondary spheres per well was counted and used to estimate the mean number of spheres per 2,000 cells. Limiting dilution assay on GICs that had been sorted for CD133 was plated at limiting dilution (from 200 to 2 cells per well) in 200 μL of TSM in quadruplicate in a 96-well plate and 0.37 intercepts calculated (27) to determine the sphere-forming frequency.

**Cell proliferation assay**

Single cells were plated in a 96-well plate at a density of 1,000 cells/200 μL per well in quadruplicate and incubated for 5 days. Twenty microliters of Alamar Blue (Invitrogen), a fluorescent cell metabolism indicator, was added to each well approximately 18 hours prior to the readout time point. Fluorescence was measured using a FLUOstar Omega Fluorescence 556 Microplate reader (BMG LABTECH) at excitation and emission wavelengths of 535 nm and 600 nm, respectively. Readings were analyzed using Omega analysis software.

**Viral production and transduction**

Lentiviral stocks shCD133-1 and shCD133-2, expressing shRNAs targeting human CD133 (5’GGCTCTTCTTATTCAGGATAT3’ and 5’GTGAAACTTACCTCAATGA3’, respectively), and the control vector, shGFP (5’ACAAACAGCCACAAGCTCTATA3’), were gifts from Dr. Jason Moffat. A CD133 overexpression vector was purchased from Genecopoeia. Replication-incompetent lentiviruses were produced by cotransfection of the expression vector and packaging vectors pMD2G and psPAX2 (for knockdown vectors) and pLP1, pLP2, and pLP/VSVG (for overexpression vectors) in HEK 293FT cells. Viral supernatants were harvested 48 hours after transfection, filtered through a 0.45-μm cellulose acetate filter, and precipitated with PEG (System biosciences). The viral pellet was resuspended in 1.0 mL of DMEM F-12 media and stored at −80°C.

**Quantitative real-time–polymerase chain reaction**

Total RNA was extracted using a Norgen Total RNA isolation kit and quantified using the NanoDrop Spectrophotometer.
ND-1000. Complementary DNA was synthesized from 0.5 to 1 μg RNA by using iScript cDNA Super Mix (Quanta Biosciences) and a C1000 Thermo Cycler (Bio-Rad) with the following cycle parameters: 4 minutes at 25°C, 5 minutes at 85°C, hold at 4°C. qRT-PCR was performed by using Perfecta SybrGreen (Quanta Biosciences) and an Opticon Chrom4 instrument (Bio-Rad). Gene expression was quantified by using Opticon software, and expression levels were normalized to GAPDH expression. Primers are listed in Supplementary Table S4.

Flow cytometric analysis and cell sorting
Tumorspheres were dissociated and single cells resuspended in PBS þ 2 mmol/L EDTA. Cell suspensions were stained with APC-conjugated anti-CD133 or a matched isotype control (Miltenyi) and incubated for 30 minutes on ice. Samples were run on a MoFlo XDP Cell Sorter (Beckman Coulter). Dead cells were excluded using the viability dye 7AAD (1:10; Beckman Coulter) or using near IR Live/Dead fixable staining kit (Life technologies). Compensation was performed using mouse IgG CompBeads (BD). Expression of CD133 was defined as positive or negative based on the analysis regions set on the isotype control. Cells were sorted into tubes containing 1 mL TSM, and small aliquots of each sort tube were reanalyzed to determine the purity of the sorted populations. Cells were allowed to equilibrate at 37°C for a few hours prior to use in experiments.

Generation of CD133 gene signature and identification of compounds for selective therapeutic targeting of GICs

Patients and samples. All data were publicly available and downloaded from the gene expression omnibus (http://www.ncbi.nlm.nih.gov/geo/) or the Repository for Brain Neoplasia Data (REMBRANDT, http://rembrandt.nci.nih.gov/). Multiple CD133-signature discovery cohorts (GSE4290, GSE7696, GSE13041) were independently evaluated to determine CD133 coexpressed genes. Together, these cohorts comprised 455 patient tumor gene expression profiles. The REMBRANDT data were used as an independent validation set. In every case, the raw intensity files (.CEL) comprising each dataset were downloaded and normalized using the Robust Multichip Algorithm (RMA) to generate probeset intensities (33).

Identification of target-related genes. CD133 signature genes were identified by their coexpression with CD133 (204304_s_at) based on a Pearson distance function (34). We filtered these results such that only probe sets appearing in the most and least 5% of coexpressed probe sets within each discovery cohort were included in the CD133 signature. In this fashion, the CD133 signature comprised overlapping probe sets among the top and bottom 5% of coexpressed with CD133 in each of the three training cohorts. The final CD133 signature comprised 65 probe sets with positive and 20 probe sets with negative correlation to CD133 transcript levels (Supplementary Table S2).

Evaluation of CD133 signature. To evaluate the target index, the expression values for each probe set were transformed such that the mean and SD were set to 0 and 1 in each dataset, respectively. A target index was calculated for each patient as follows:

\[ \text{Index} = \frac{\sum_{i=1}^{n} \text{expression}_i}{n} - \frac{\sum_{i=1}^{n} \text{CD133 expression}_i}{n} \]

where \( x \) is the transformed expression, \( n \) is the number of probe sets, \( P \) is the set of probes with reported positive correlation to the target probe set, and \( N \) is the set of probes with reported negative correlation to the target probe set (35, 36). Patients were stratified into either CD133 high or CD133 low groups based on median CD133 signature score.

Gene set enrichment analysis. Gene set enrichment analysis (GSEA) was performed using the gene expression profiles of REMBRANDT tumor samples as previously described, using previous defined embryonic stem (ES) cell gene sets (37), and the CD133 signature to define phenotype.

Connectivity mapping. Connectivity mapping was carried out using the Connectivity Map 02 (https://www.broadinstitute.org/genome_bio/connecitivitymap.html; ref. 38). Probe sets that were among the top 5% coexpressed or anti-coexpressed genes in at least 2 of the 3 discovery cohorts were used as tags to identify perturbagens that reduce or increase the expression of CD133 coexpressed and anti-coexpressed genes, respectively.

Network analysis. Probe sets among the top 5% CD133 coexpressed probe sets were mapped as genes into nodes of the REACTOME functional interaction network (refs. 39, 40; Supplementary Table S5). Next, Pearson correlation coefficients were calculated for all interacting gene pairs and assigned as edges onto the network. Markov clustering was used to subset the network and identify modules of interacting genes. Subsequently, modules were annotated with significantly enriched pathways. All network analyses were carried out using Cytoscape (v2.8.2) and the Reactome Fls plugin (v2012).

Targeted treatment of GICs
IC\(_{50}\) values were determined as follows: 1,000 cells (unsorted and CD133-sorted) were plated in a 96-well plate in quadruplicates at a volume of 200 μL/well in increasing concentrations (5 nmol/L–5 μmol/L) of hit compounds (Supplementary Table S3) including pyrvinium. DMSO was used as a control. Seven days after treatment, an alamar blue assay was performed as described in the proliferation assay. Dose–response curves were fitted to the data. To determine the tumor-initiating function of treatment-refractory cells, GICs were treated with pyrvinium at its IC\(_{50}\) levels for 3 days in vitro prior to intracranial injections in NOD-SCID mice. Trypan blue exclusion was used to count viable cells using the Countess Automated Cell Counter (Invitrogen).

In vivo GIC intracranial injections and H&E staining of xenograft tumors
Intracranial injections were performed as previously described (6) using each of the following GICs: shGFP, shCD133, Ctrl OE, CD133 OE, DMSO-treated control, and pyrvinium-treated. Briefly, the appropriate number of live cells (determined by Trypan Blue exclusion) was resuspended in 10 μL of PBS. NOD-SCID mice were anaesthetized using isofluorane gas (5% induction, 2.5% maintenance), and cells were injected into the frontal lobe using a 10 μL Hamilton syringe as per Research Ethics Board (REB)-approved protocols, in a nonrandomized, nonblinded fashion. The mice injected with drug-treated cells were sacrificed when the control group reached endpoint. Upon reaching endpoint, brains were harvested, formalin-fixed, and paraffin-embedded for hematoxylin and eosin (H&E) and human COX IV staining (Cell Signaling).
Microarray analysis
RNA samples from 3 independent GIC lines that were treated with 200 nmol/L pyrvinium or DMSO were labeled using the Illumina Total Prep-96 RNA Amplification Kit (Ambion) as per amplification protocol. cRNA (750 ng) generated from these samples was hybridized onto Human HT-12 V4 Beadchips. The BeadChips were incubated at 58°C, with rotation speed 5 for 18 hours for hybridization. The BeadChips were washed and stained as per Illumina protocol and scanned on the iScan (Illumina). The data files were quantified in GenomeStudio Version 2011.1 (Illumina). All samples passed Illumina sample-dependent and -independent QC Metrics. GSEA analysis was performed using the MySigDB oncogenic signature collection.

Wnt/TCF reporter assays
GBM GICs and GBM cells transduced with shGFP, shCD133-1, OE Ctrl, OECD133 vectors were cotransfected with the constructs 8X TOPFlash (1.8 μg), driving firefly luciferase (41), and pRL-CMV (0.2 μg), driving expression of renilla luciferase for normalization (Promega). After 24 hours, GBM cells were supplemented with Wnt 3a conditioned media with or without pyrvinium (200 nmol/L), and KD and OE cells were supplemented with TSM media. Cells were washed twice with PBS 24 hours following media change and lysed with passive lysis buffer (Promega). The luciferase reporter activities were measured using a luminometer as per the manufacturer’s instructions (Promega Dual-Light System).

Statistical analysis
Biologic replicates from at least three patient samples were compiled for each experiment, unless otherwise specified in figure legends. Respective data represent mean ± SD, n values are listed in figure legends. Student t test analyses, 2-way ANOVA with Bonferroni post-hoc tests, and log-rank (Mantel–Cox test) analysis were performed using GraphPad Prism 5. P < 0.05 was considered significant. Statistical tests for in silico analyses were two-sided and were completed in R.

Results
CD133 expression and increased self-renewal are prognostic variables in human GBM
We have shown that a higher self-renewal index correlates with reduced GBM patient survival (42). Here, we sought to establish the clinical utility of CD133 as a prognostic GBM biomarker. Using a median cutoff of 15.8% based on CD133 expression in 23 primary human GBMs (Supplementary Table S1), we compared overall survival in CD133high (n = 11) with CD133low (n = 12) GBMs. CD133high tumors were associated with a lower survival (P = 0.012; Supplementary Fig. S1A) in keeping with their higher self-renewal index. The median survival of CD133high and CD133low tumors was 10 and 14.5 months, respectively. Recent reports suggest that CD133 is a prognostic biomarker for relapse (21), time to malignant progression from low-grade gliomas, and poor survival (22). To provide a functional context for the clinical utility of CD133 expression in GBM, we enumerated GIC frequency through in vitro sphere formation assays. When compared with CD133− cells, CD133+ cells generated larger (Supplementary Fig. S1B), more frequent (Supplementary Fig. S1C and S1D), and more proliferative (Supplementary Fig. S1D) GBM tumorspheres. Thus, CD133+ cells may promote poor outcome through an enhanced self-renewal mechanism.

CD133 functions to regulate GIC self-renewal
As CD133 serves as a prognostic biomarker, we aimed to determine its function in gliomagenesis by short hairpin RNA (shRNA)-mediated silencing approach. In order to exclude the possibility of off-target effects, we used two independent shRNA vectors. Both constructs yielded efficient knockdown (KD) of mRNA levels (Fig. 1A), and shCD133-1 was more effective in reducing the protein levels (Fig. 1B). We performed self-renewal and proliferation assays to assess the effects of CD133 KD in our GIC lines. Rates of secondary tumorsphere formation (Fig. 1C) and proliferative potentials (Fig. 1D) were markedly impaired following CD133 KD with shCD133-1. We used in vivo xenotransplantation assays to assess the effect of knockdown of CD133 on tumor size. We injected shGFP and shCD133-1 cells from GBMs to assess in vivo effects of CD133 KD. Intracranial injections of shGFP GICs into NOD-SCID mice yielded invasive, multifocal tumors, whereas shCD133-1 GICs generated noninvasive, well-circumscribed lesions (Fig. 1E). Despite these differences between CD133 KD and control tumors, both conditions formed tumors, which may reflect residual CD133 from incomplete knockdown. However, the tumor size was significantly reduced upon knockdown of CD133 (Fig. 1F). Previous studies (6) together with our KD data implicate CD133 as a regulator of GIC self-renewal, a function with clinical utility beyond that of a biomarker (43).

A CD133 gene signature is predictive of poor overall glioma survival
As high fractions of CD133+ tumor cells negatively correlate with patient survival, and CD133 positively influences GIC self-renewal, we hypothesized that the transcriptional program linking CD133 with GBM stemness could be used to identify novel therapeutic targets (44). We generated a CD133 gene expression signature based on CD133 coexpressed and anti-coexpressed genes from 3 independent brain tumor gene expression datasets (GSE4290, GSE7696, GSE1304) representing 455 gliomas (Fig. 2A). The CD133 signature comprised probe sets from the top and bottom 5% of all probes based on similarity in expression to CD133 (Fig. 2B and C; Supplementary Table S2). To confirm the capacity of the CD133 signature to measure GBM stemness, we reasoned that the signature should identify tumors enriched in stem cell processes, and display an aggressive course of disease. Initially, we completed GSEA (45) with previously defined ES cell gene sets (37) in a validation cohort of brain tumors (REMBRANDT, n = 276; ref. 46; Supplementary Fig. S2). REMBRANDT tumors with a CD133high signature, defined using a median cut-point for the CD133 signature score, were significantly enriched for each ES gene set. We also compared signature values across all REMBRANDT gliomas and found that CD133 signature values were significantly higher in GBM relative to low-grade gliomas (Fig. 2D, ANOVA). We compared the survival of all glioma patients (n = 238) with either CD133high or CD133low signature tumors using the median signature score as a cutoff for patient stratification. Patients with CD133high tumors displayed dramatically poorer overall survival relative to CD133low patients (Fig. 2E; HR = 2.1, P < 0.0001), a finding
Figure 1.
CD133 knockdown impairs the self-renewal and tumor-initiating capacity of GICs. CD133 (A) transcript by qRT-PCR [bars represent the mean relative transcript level of 3 technical replicates of a single sample (one-way ANOVA)] and (B) protein levels as shown by flow cytometric analysis are significantly reduced following shRNA-mediated knockdown of CD133. CD133 knockdown functionally diminishes the (C) self-renewal by secondary sphere formation assay (left: bars represent the mean spheres per 2,000 cells of 8 technical replicates of a single sample, mean ± SD, two-tailed t test), and (D) proliferative potential by Alamar Blue proliferation assay (left: bars represent the mean fluorescence intensity of 3 technical replicates of a single sample; mean ± SD; two-tailed t test); E, BT428-generated xenograft tumors demonstrate a significant reduction in tumor size (red arrows) and number of mitotic cells identified by thickening of chromatin (blue arrows) following CD133 knockdown (bottom panel) when compared with control shGFP-generated xenografts (top). F, shCD133-1 xenograft tumors were significantly smaller when compared with shGFP-generated xenografts. A.U., arbitrary units; scale bar: left = 2 mm, right = 200 μm. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
replicated in the GBM patient subgroup (Fig 2F; HR = 1.5, P = 0.02). While our signature predicted poor survival, we wanted to assess whether this was in part due to treatment resistance conferred by a CD133 transcriptional profile. MGMT promoter methylation status serves as a robust biomarker for sensitivity to temozolomide, the primary chemotherapy for GBM patients. Among a cohort of GBMs with methylation status data (GSE7696, n = 80 GBM), the CD133<sup>high</sup> signature was significantly elevated in patients whose tumors had unmethylated MGMT (Fig. 2G) and was associated with a poor overall outcome (Fig. 2H, tertile cut-point). Collectively, these analyses demonstrate a robust relationship between the CD133
signature, tumor aggressiveness, and GIC transcriptional pathways.

A CD133-dependent gene signature identifies molecular networks for therapeutic targeting

To identify key targetable biologic processes associated with CD133 gene expression and GIC-intrinsic pathways, we generated a protein interaction network (Fig. 3A) comprising protein products encoded by CD133 coexpressed genes. CD133 coexpressed genes interacted in 4 subnetworks or modules (Fig. 3B–E), each associated with distinct biologic processes (Fig. 3F). Modules 0 (Fig. 3B), 1 (Fig. 3C), 2 (Fig. 3D), and 3 (Fig. 3E) were enriched in cell proliferation pathways (including both PLK1 and Aurora B kinase), RNA processing and metabolism, protein translation and export, and DNA repair (Fanconi anemia pathway), respectively. These pathways represent diverse biologic programs associated with CD133 gene expression and GIC maintenance that may contribute to the treatment-refractory nature of GBM. To identify additional candidates specific to GIC self-renewal machinery, we completed connectivity mapping (38) with CD133 coexpressed and anti-coexpressed genes. With data on 6,100 chemical perturbations on gene expression profiles of multiple human malignancies, the Connectivity Map provides access to a high-throughput exploration of interactions between small molecules and transcriptional profiles. In searching for compounds that reduced the expression of CD133 coexpressed genes and increased the expression of CD133 anti-coexpressed genes, we identified 70 compounds, 15 of which cross the blood–brain barrier (Fig. 3G, Supplementary Table S3) and have already been reported to target TIC self-renewal (47–49).

Pyruvinium targets CD133+ cells to attenuate self-renewal

Self-renewal and proliferation assays in three GBMs showed that the majority of our hits were unable to target properties of stemness and thus resembled current chemotherapeutic agents (Supplementary Fig. S3A and S3B). Of our candidate compounds, pyruvinium, an FDA-approved anthelminthic drug, functioned as the most potent inhibitor of self-renewal and proliferation (Supplementary Fig. S3A). Given our data implicating CD133 in regulation of self-renewal, we treated CD133high and CD133low GBMs with pyruvinium and observed a significantly lower IC50 in CD133high GICs than in CD133low GICs (Fig. 4A). Pyruvinium has shown potent toxicity against various cancer cell lines (50, 51), but its effects on TIC populations are untested. Thus, we sorted our GIC lines for CD133+ and CD133− fractions to assess the effects of pyruvinium compared with phenothiazine drugs, which demonstrated effects on cell proliferation and self-renewal in our unsorted GIC lines (Supplementary Fig. S3A). Phenothiazines showed minimal selectivity toward CD133− cells and did not target CD133+ cells at a therapeutic dose (Supplementary Fig. S3B). By contrast, pyruvinium targeted the self-renewal (Fig. 4B), proliferative capacities (Fig. 4C) and caused cell death (Supplementary Fig. S4A) of CD133+ cells in the low nanomolar range and CD133− cells at a slightly higher dose, distinguishing this drug from the majority of chemotherapeutic agents, which completely fail to target the cancer stem cell population at a clinically relevant dose.

Notably, our GIC lines were completely resistant to temozolomide (TMZ), current first-line chemotherapy for GBM (Supplementary Fig. S3A). As pyruvinium showed the ability to target CD133+ GICs in primary GBMs, we aimed to test its efficacy in recurrent GBMs driven by treatment-refractory CD133+ cells. Pyruvinium also targeted CD133+ cells at a lower dose in a recurrent GBM (Supplementary Fig. S4B). We also found that there was a decline in CD133+ cells after pyruvinium treatment in both primary and recurrent GBM samples (Fig. 4D). To assess the clinical utility of treating GBMs with pyruvinium, CD133high GIC lines were treated with pyruvinium at IC50 or DMSO after which 1 × 10⁷ viable cells, representing treatment-refractory CD133+ GICs, were injected intracranially into NOD-SCID mice. Pyruvinium-treated mice displayed no evidence of tumor formation, suggesting complete abrogation of the self-renewal machinery required for tumor initiation (Fig. 4E). By contrast, DMSO-treated control mice exhibited large, infiltrative GBM tumors (Fig. 4E). These pathologic differences were also reflected in a significant survival advantage for the pyruvinium-treated cohort (Fig. 4F). Together, these data implicate pyruvinium in preventing GIC-driven recurrence.

Pyruvinium targets the dynamic flux of CD133 expression in GICs by inhibiting developmental signaling pathways

TICs likely exist in a steady-state equilibrium with TMCs (tumor-maintaining cells), preserving the cellular diversity of solid tumors (16). Because CD133 expression changes during tumor evolution (30), we sought to investigate how ectopic expression of CD133 in CD133-naïve cells affects the phenotypic hierarchy in GBM. Transgenic overexpression of CD133 in three GIC lines resulted in a marked increase in CD133 expression (Supplementary Fig. S5A and S5B), with corresponding increases in self-renewal (Fig. 5A) and proliferation (Fig. 5B; ref. 52).

We then aimed to determine the functional consequence of transgenic CD133 overexpression on self-renewal. We detected a significant increase in CD133 expression in a GIC line (BT241) with low CD133 (≤3% CD133+ cells; Fig. 5C). Ectopic expression of CD133 in CD133low BT241 cells significantly increased self-renewal (P < 0.0001; Fig. 5D) and proliferative potential (P < 0.0001; Fig. 5E) of these cells when compared with controls. Given glioma cell plasticity (53, 54), we aimed to model the emergence of CD133 from CD133− cells and the consequences to drug treatment. Upon converting CD133low BT241 (Ctrl OE) cells to CD133+ GICs, we treated our transformed cells (CD133 OE) with pyruvinium. CD133 OE cells demonstrated preferential sensitivity to pyruvinium, illustrated by a reduced capacity for secondary sphere formation when compared with Ctrl OE cells (Fig. 5F). Notably, the IC50 and therapeutic dose of pyruvinium declined to 120 nmol/L in CD133 OE cells from 240 nmol/L in Ctrl OE cells (Fig. 5G), suggesting an increased potency in CD133-expressing cells. The differential sensitivity of CD133 OE to pyruvinium was much more pronounced at lower concentrations of 25 and 50 nmol/L whereas no significant difference was observed at higher concentrations (Fig. 5H). Together, these data suggest that pyruvinium may constitute a unique therapy for cells endowed with increased CD133-driven stemness.

We have shown that pyruvinium functions as a novel inhibitor of self-renewal in CD133+ GICs. Recent studies have suggested that pyruvinium exerts its potent antineoplastic effects by attenuating developmental signaling pathways such as Wnt (55) and sonic hedgehog (Shh; ref. 30). CD133 has been shown to form a ternary complex with HDAC6 to stabilize the central molecule of the canonical Wnt pathway, β-catenin, and thereby promote Wnt target gene activation (24). We hypothesized that pyruvinium may...
function as an anti-GIC agent by inhibiting Wnt/β-catenin signaling. Using the well-characterized Wnt inhibitor XAV939, which stimulates β-catenin degradation via Axin stabilization (56), we compared the levels of the β-catenin target gene, Axin2, in GICs treated with pyrvinium and XAV939. Both treatment groups yielded equivalent and significant decreases in Axin2 levels.

Figure 3.
A CD133 gene signature identifies key transcriptional pathways for GIC-targeted therapy. A, a protein interaction network based on the protein products encoded by CD133 coexpressed genes identified 4 sub-networks or modules. Modules (B) 0, (C) 1, (D) 2, and (E) 3 were enriched in (F) cell proliferation pathways (including both PLK1 and Aurora B kinase), RNA processing and metabolism, protein translation and export, and DNA repair (Fanconi anemia pathway), respectively. G, connectivity mapping based on the CD133 protein interaction network identified 15 compounds that reduced and increased the expression of CD133 coexpressed and anti-coexpressed genes, respectively.
Figure 4.
Pyrvinium selectively impairs the self-renewal machinery of CD133+ cells. A, GBM samples with a CD133high fraction (BT428A, BT459; IC50s: 12.89 nmol/L and 29.82 nmol/L) are much more sensitive to pyrvinium treatment than those with a CD133low fraction (BT241, BT486; IC50s: 239.8 nmol/L and 122.5 nmol/L, respectively). 2-tailed t test P < 0.001. B, self-renewal of the CD133+ cell population in four distinct GBM samples, as evaluated by secondary sphere formation assay, is selectively reduced following treatment with pyrvinium. Data are presented as mean ± SD. C, proliferative potential of the CD133+ cell population in four distinct GBM samples, as assessed by Alamar Blue proliferation assay, is selectively reduced following treatment with pyrvinium. D, CD133+ cells decline upon treatment with 200 nmol/L treatment with pyrvinium for 48 hours in both primary (BT428) and recurrent (BT 566) GBM samples. E, xenografts (H&E, Cox IV staining for human cells) generated from pyrvinium-treated cells displayed no visible tumor suggesting pyrvinium as an inhibitor of self-renewal and tumor initiation. F, mice injected with pyrvinium-treated cells maintain a significant survival advantage over control mice (n = 4, P = 0.04). Bars represent the self-renewal or proliferative potential as per the mean fluorescence intensity of 8 or 3 technical replicates (mean ± SD), respectively, normalized to DMSO control. Two-way ANOVA with Bonferroni post-hoc tests were performed to assess significance. n.d., not determined. Scale bar, 2 mm. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure 5.

CD133 overexpression enhances self-renewal capacity of CD133- cells, sensitizing them to pyrvinium treatment. CD133 OE functionally enhances the (A) self-renewal as evaluated by secondary sphere formation assay (left: mean spheres per 2,000 cells, n = 10) and (B) proliferation quantified by Alamar Blue cell viability reagent (left: mean fluorescence intensity, n = 3). C, representative flow cytometric plot showing an increase in CD133+ cells (from 2.6% to 50.0%) following CD133 OE (BT241). CD133 OE functionally enhances the (D) self-renewal as assessed by secondary sphere formation assay [mean spheres formed per 2,000 cells: 16.25 ± 4.199 (Ctrl OE) and 155.0 ± 9.258 (CD133 OE)] and (E) proliferation determined by Alamar Blue cell viability reagent [mean fluorescence intensity: 12188 ± 1204 (Ctrl OE) and 71669 ± 274.4 (CD133 OE)]. F, impaired sphere formation in CD133 OE cells compared with control following pyrvinium treatment: 92.50 ± 24.05% (Ctrl OE) and 69.10 ± 33.84% (CD133 OE). G, IC50 for pyrvinium-treated CD133- cells decline from 240 nmol/L (Ctrl OE) to 120 nmol/L (CD133 OE). H, pyrvinium treatment is more effective in targeting CD133+ cells at lower concentrations (25 nmol/L, P < 0.001; 50 nmol/L, P < 0.01; 100 nmol/L, P < 0.05; 200 nmol/L, P < 0.1).
relative to their controls ($P > 0.05$; Fig. 6A), indicating the canonical Wnt/β-catenin pathway as a possible target of pyrvinium treatment. We also found that pyrvinium reduced Wnt/TCF reporter activity (8X TOPFlash; Fig. 6B), further confirming that Wnt/β-catenin pathway to be a potential target of pyrvinium. To elucidate Wnt activity in CD133 sorted populations, we assessed Axin2 expression levels in multiple GIC samples and found them to be elevated in CD133$^+$ cells when compared with CD133$^-$ cells (Fig. 6C). We used Wnt/TCF reporters on GICs that were KD or OE CD133 and found that reporter activity was reduced in cells that were knocked down for CD133 (Fig. 6D) and was enhanced in cells that were overexpressing CD133 (Fig. 6E). This suggests a link between Wnt/β-catenin pathway and CD133. To understand additional mechanisms by which pyrvinium targets GICs, we performed global gene expression profiling of pyrvinium-treated GICs. GSEA revealed the most significant reduction in pathways that govern self-renewal and proliferation: Bmi1, JAK2, and Wnt/β-catenin (Fig. 6B, Supplementary Fig. S6). These data further establish CD133 as a GIC self-renewal gene responsible for endowing cells with a stemness phenotype that may be selectively targeted with pyrvinium, at least in part through inhibition of the canonical Wnt pathway.

**Discussion**

The cellular composition of solid tumors reflects a complex ecosystem in which cells adapt, interact, and compete for survival. Our results establish CD133 as an important factor that can enable glioma cells to adapt to current therapies and transit between different clonal populations of tumor cells based on...
phenotypic differences in stemness. Our approach for the treatment of human GICs takes into account these functional properties endowed by CD133 expression. In linking the core transcriptional program associated with CD133 and GBM stemness, our prognostic GIC signature not only enriched for those patients most likely to relapse rapidly, but also presented us with a phenotypic platform for assessing the efficiency with which candidate molecules impaired the self-renewal of GICs. Whereas the majority of our small molecule hits were unable to target CD133, pyrvinium uniquely inhibited the self-renewal of CD133+ cells, and lead to a survival advantage in an in vivo model of recurrence. If current chemotherapeutics fail to eliminate cancer stem cell populations that evade therapy to drive patient relapse, pyrvinium will provide a unique survival advantage by targeting both the CD133+ bulk tumor and CD133+ GICs. In describing CD133 as a functionally relevant molecule amenable to therapeutic targeting, this work provides an approach for eradicating other GIC populations responsible for disease progression and relapse.

In the evolution of GBM, CD133 may mark the original TIC that seeds a tumor. A low-frequency CD133+ subclone may then persist throughout the course of treatment by generating a cellular hierarchy that contributes to intratumoral heterogeneity and the acquisition of drug resistance. Whereas current chemotherapeutic agents may debulk the bottom of the cellular hierarchy, cells endowed with a CD133-driven self-renewal phenotype could escape such therapies and re-emerge to initiate tumor relapse. A similar paradigm in acute myeloid leukemia describes rare hematopoietic stem cells that acquire preleukemic mutations that are able to reconstitute the heterogeneous leukemic landscape and maintain a clonal reservoir of treatment-resistant cells implicated in leukemic progression and relapse (57). The presence of these ancestral leukemic cells at disease onset and recurrence highlights self-renewal as an essential process by which these cells persist throughout tumorigenesis. Given that self-renewal is largely measured by functional assays that require proliferation, the identification of targeted therapies that affect proliferation, the identification of targeted therapies that affect both self-renewal and proliferation has been increasingly difficult (43, 47). This has been especially true in GBM where self-renewal regulators identified in transgenic mouse models have been of limited clinical utility (58).

Mechanistically, CD133 may modulate proliferation through activation of the PI3K/AKT pathway as CD133 phosphorylation regulates a direct interaction with p85, the regulatory subunit of PI3K (23). By forming a stabilization complex with β-catenin, CD133 may also activate targets of Wnt/β-catenin signaling to maintain the self-renewal capacity of GICs (24). As a functional nexus for the proliferation and self-renewal of GICs, CD133 presents a potential high-yield therapeutic target. Our data establish pyrvinium as a novel compound capable of attenuating CD133-mediated proliferation, self-renewal, and tumor formation in GBMs. Pyrvinium has been shown to downregulate both Akt and Wnt signaling, although its effects on β-catenin signaling output may be secondary to its attenuation of Akt phosphorylation (59). Our gene expression data additionally show pyrvinium may also abrogate signaling through other pathways, including Bmi1 and Jak2. Importantly, our lab has previously identified Bmi1 as an essential regulator of GIC self-renewal in CD133+ cells (42). Therefore, although CD133 may be the functional hub for GICs, pyrvinium may form a therapeutic nexus of its own with combinatorial targets that are downstream of one another.

Admittedly, many of the compounds identified in our CD133 signature-based connectivity map screen did not target GICs, suggesting that our approach may be limited. This could be a result of inherent limitations within the connectivity map that uses cell lines, whereas we completed experiments with primary cells. Moreover, the connectivity map does not include any brain tumor cell lines, suggesting that ‘hit’ compounds may be less relevant in brain tumor models. Finally, it is also possible that many of the candidates do indeed target GICs, but that the sphere-forming assay does not optimally detect all GIC targeting compounds. Notably, many of our candidates comprised phenothiazines, which have previously been reported to target cancer stem cells (CSCs) (47). We also note that although the screen identified false positives, the overall true hit rate was excellent. We screened some 20 compounds in order to discover that pyrvinium displays anti-GIC activity, representing an approximate 5% hit rate. In contrast, many groups have screened 1000s of compounds to identify a single or a few compounds that target TICs. For example, Gupta and colleagues report screening approximately 16,000 compounds to identify salinomycin as an antitumor breast cancer TIC therapeutic (60).

The failure of current cancer therapeutics may be attributed to a number of determinants such as clonal expansion based on cellular and genomic diversity, properties of stemness such as self-renewal, and the inability to effectively identify targets that act on multiple pathways with functional importance. Our novel drug discovery approach can be applied to other prospectively identified GIC populations that drive tumor heterogeneity. Our study provides a strategic platform for the preclinical evaluation of these factors in primary human cancer cells. The eradication of TICs is dependent on the translation of preclinical findings to the patient bedside, so future studies should be focused on the identification of additional signaling hubs that regulate the self-renewal of human TICs. Compounds that converge on these cell-intrinsic pathways may overcome the dynamic nature of TICs and thereby prevent the evolution of TIC clones that drive tumor initiation, maintenance, and relapse.
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Chitra Venugopal, Robin Hallett, Parvez Vora, et al.


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