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Vulnerability of Glioblastoma Cells to Catastrophic Vacuolization and Death Induced by a Small Molecule

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SUMMARY

Glioblastoma multiforme (GBM) is the most aggressive form of brain cancer with marginal life expectancy. Based on the assumption that GBM cells gain functions not necessarily involved in the cancerous process, patient-derived glioblastoma cells (GCs) were screened to identify cellular processes amenable for development of targeted treatments. The quinine-derivative NSC13316 reliably and selectively compromised viability. Synthetic chemical expansion reveals delicate structure-activity relationship and analogs with increased potency, termed Vacquinols. Vacquinols stimulate death by membrane ruffling, cell rounding, massive macropinocytic vacuole accumulation, ATP depletion, and cytoplasmic membrane rupture of GCs. The MAP kinase MKK4, identified by a shRNA screen, represents a critical signaling node. Vacquinol-1 displays excellent in vivo pharmacokinetics and brain exposure, attenuates disease progression, and prolongs survival in a GBM animal model. These results identify a vulnerability to massive vacuolization that can be targeted by small molecules and point to the possible exploitation of this process in the design of anticancer therapies.

INTRODUCTION

Tumor development involves mutations that can be gain-of-function mutations in proto-oncogenes or loss-of-function mutations in tumor suppressor genes that lead to fundamental changes in the biology of the cell, resulting in cancer. Genomic studies of hundreds of glioblastoma multiforme (GBM) samples have led to a comprehensive insight into the genomic landscape of GBM, revealing both gain- and loss-of-function in core signaling pathways commonly activated, including the receptor tyrosine kinase (RTK/RAS) oncogenic pathway with alterations in *EGFR/PDGFRA/PI3K/PTEN/NF1/RAS*, the p53 pathway with changes in *TP53/MDM2/MDM4/p14ARF*, and finally, the cell-cycle regulatory pathway with alterations in *RB1/CDK4/p16INK4A/CDKN2B*, with most GBM tumors having genetic alteration in all three pathways (Chen et al., 2012b; Furnari et al., 2007; Parsons et al., 2008; Brennan et al., 2009, 2013; Verhaak et al., 2010; Cancer Genome Atlas Research Network, 2008). The consequence is a fueling of cell proliferation and enhanced survival and invasion properties, while preventing tumor cells from apoptosis and activation of cell-cycle checkpoints. Consistently, malignant gliomas are among the most devastating human cancers (Louis et al., 2007). GBM is essentially incurable, even when aggressive therapies based on surgical tumor resection and concomitant chemotherapy and radiotherapy are implemented (Stupp et al., 2005), and only 3%–5% of patients survive longer than 3 years due to disease recurrence (Dolecek et al., 2012).

The small population of GBM cells with stem/progenitor cell characteristics (Hemmati et al., 2003; Singh et al., 2003, 2004) seed growth of new tumors (Gallo et al., 2013; Pollard et al., 2009; Singh et al., 2003, 2004) and drive malignancy, metastasis, and tumor recurrence, promoting resistance against radiation-based therapy (Bao et al., 2006) and chemotherapy (Bleau and Holland, 2009; Chen et al., 2012b). These tumor-initiating cells are believed to be relatively quiescent (Alcantara Llaguno et al., 2009; Barami et al., 2009), which could contribute to disease recurrence following current therapeutic strategies targeting intracellular processes associated with cell division.

Unlike several other forms of cancer in which identification of participating gene products have resulted in series of drugs

neutralizing the function gained by the genetic alterations, the complexity and diversity of glioblastoma genetics have prevented a simple strategy for therapeutic targeting. New approaches focused on neutralizing abnormalities underlying tumor development have only had limited success so far (Dent et al., 2008; Polivka et al., 2012). We reasoned that gene products causing glioma are part of signaling pathways that are involved in diverse cellular functions. Therefore, gain- and loss-of-function mutations would be predicted to lead to acquired functions also in cellular properties not necessarily involved in cell transformation and proliferation. Hence, there should be unique cellular properties of glioma that are low or absent in other cell types. If identified, such features can be exploited for development of conceptually new strategies of therapy. In an unbiased phenotypic screen using a diversity set of small molecules on glioblastoma cells (GCs), a biological process resulting in a robust loss of viability and increased cytotoxicity was discovered. GCs are vulnerable to an MKK4-dependent signaling inducing catastrophic vacuolization and cell death.

RESULTS

Induction of Rapid and Specific Cell Death of Glioma Cells by a Small Molecule

In order to identify pathways vulnerable for targeted treatment of GCs, a phenotypic screen was performed to identify compounds active on GCs without affecting embryonic stem cells or human fibroblasts. GC cultures were independently generated from two cases of GBM, according to Pollard et al. (2009) which allows for adherent growth of cells with tumor-initiating and stem-like properties. These cell lines, designated U3013MG and U3047MG, were screened, rescreened, and confirmed using 1,364 compounds of the NIH diversity set II for phenotypic changes observed following phalloidin staining (Figure 1A and Figure S1A available online). 234 compounds showed effects after 2 days with principal phenotypes observed designated as tiny (T, dead or reduced cell size) or loose or fuse (LOF, senescence-like morphology), respectively (Figures 1B–1D). Filter screens against mouse embryonic stem cells (mESCs) and human fibroblasts reduced the initial 234 compounds to 63 with effects selective for GCs (Figures 1A and S1B). The compounds were confirmed active on U3013MG and U3047MG GCs, as well as on seven other patient-derived GC cultures that were established (U3024MG, U3017MG, U3031MG, U3037MG, U3086MG, U3054MG, and U3065MG; Figures 1A and S1E). The 63 compounds (Table S1) were examined in a recovery assay (Figures S1C and S1D) by quantification of cytotoxicity, apoptosis, and cell viability (Figure S2A) in U3013MG GCs and human fibroblast cells, as well as cell-cycle analysis by flow cytometry (Figure S2B). In the recovery assay, 2 days with compound were followed 2 more days without compound. Only 12 compounds had an irreversible effect at the same concentration that caused the acute effects (Figures S1C and S1D). Quantification of cell viability (ATP) and cytotoxicity (CytoTox-Glo) of the 63 compounds identified partly the same hit compounds (i.e., E5, E10, and A7) as most efficacious with limited effects on fibroblasts (Figure S2A). Based on these analyses and in silico absorption, distribution, metabolism, and excretion (ADME) prediction (Figures S3A–S3J), a refined list of 17 hits (Table S2) was pursued using several parameters, including cell-type-selective in vitro and in vivo efficacy, Ca^{2+} imaging, and toxicity tests. A hanging drop-based mixed culture procedure was developed for assaying selectivity (Figures S4A and S4B). U3013MG GCs and fibroblasts labeled with cell tracker red or green fluorescent dye, respectively, in cocultures confirmed selectivity (Figure S4B). Only one compound (D1) induced Ca^{2+} flux in GCs (Figures S4C–S4E). To measure toxicity, increasing concentrations of the 17 hits administered to the water of 10 days postfertilization (dpf) zebrafish embryos revealed that, whereas B6, B11, E5, E9, C2, C7, and D12 did not affect zebrafish development, embryos died, decayed, or displayed yolk edema in the presence of the remaining hits (Figure S5A). Cardiovascular toxicity of ex vivo adult zebrafish hearts (Kitambi et al., 2012) was observed for four hits (A2, A6, C9, and E9) whereas small or no effects were found on the remaining compounds (Figure S5B). In vivo efficacy was examined in a zebrafish GBM model that was developed where 3,000 U3013MG GCs labeled with cell tracker red were injected intracranially into the ventricle of 48–52 hpf larvae. The 17 hits administered to the egg water at the lowest effective in vitro cytotoxic concentration and monitoring tumor development 10 days later revealed that B7, C2, and E5 markedly reduced tumor size, with E5 being most potent (Figure S5C). Based on these analyses, further studies were focused on compound E5, which we name Vacquinol-1 due to its quinoline-alcohol scaffold (Figure 1E). Vacquinol-1 displayed high cytotoxicity (15 μM, 12 hr; Figure 1F), led to a complete loss of viability as measured by ATP depletion (Figure 1G), and selectively targeted GCs in mixed cocultures with human fibroblasts (Figures 1H and 1I). Vacquinol-1 did not affect mESCs, human fibroblasts, or osteosarcoma cells (Figures 1J–1O) but rapidly reduced the proportion of GCs in S and G2/M cell-cycle phases (7.5 μM, 12 hr; Figure 1P). Cell density had only minor effects on viability in dose response assays (Figure 1Q). The median inhibition concentration of 50% (IC_{50}) was 2.36 μM (3,000 cells/cm^2, 24 hr, Figure 1R) as compared to 139 μM by temozolomide (Figure 1S, 48 hr), a commonly used drug for treating glioma. The IC_{50} of Vacquinol-1 remained largely similar at 2, 3, and 4 days of incubation (Figures 1T–1V). Mouse glia and neuron cultures showed an IC_{50} of 15 and 29 μM, respectively (24 hr, Figures 1W and 1X), and fibroblasts showed an IC_{50} of 18.7 μM and 23 μM (24 and 96 hr, respectively; Figures 1Y and 1Z). The IC_{50} of fibroblasts was not affected by changing to GC culture medium during Vacquinol-1 treatment (Figures S1F and S1G). Furthermore, Vacquinol-1 was ineffective on bladder, prostate, breast, and neuroblastoma cancer cell lines (Figure S1H).

Induction of a Catastrophic Unconventional Cell Death Pathway by Vacquinol-1

Apoptosis is associated with a rapid loss of ATP. Vacquinol-1 administration (7.5 μM, 7 hr) led to a marked and significant increase of dead cells analyzed by flow cytometry, similar to staurosporin (1 μM, 7 hr; Figures 2A–2F). The apoptosis inhibitor Q-VAD only modestly rescued cells from death at 3 and 7 hr (Figure 2F). Active cleaved Caspase-3 was not increased by Vacquinol-1 as compared to vehicle-treated cultures, whereas doxorubicin (10 μM) markedly increased positive cells