Impairment of Glioma Stem Cell Survival and Growth by a Novel Inhibitor for Survivin–Ran Protein Complex

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Abstract

Purpose: Glioblastoma multiforme (GBM) is a devastating disease. Recent studies suggest that the stem cell properties of GBM contribute to the development of therapy resistance.

Experimental Design: The expression of Survivin and Ran was evaluated by immunohistochemistry with GBM tissues, and quantitative reverse transcriptase (qRT)-PCR and immunocytochemistry with patient-derived GBM sphere cultures. With a computational structure-based drug design, 11 small-molecule compounds were designed, synthesized, and evaluated as inhibitor candidates for the molecular interaction of Survivin protein. The molecular mechanism of the lead compound, LLP-3, was determined by Western blot, ELISA, in situ proximity ligation assay, and immunocytochemistry. The effects of LLP-3 treatment on GSCs were evaluated both in vitro and in vivo. Quantitative immunohistochemistry was carried out to compare Survivin expression in tissues from 44 newly diagnosed and 31 recurrent post-chemoradiation GBM patients. Lastly, the sensitivities of temozolomide-resistant GBM spheres to LLP-3 were evaluated in vitro.

Results: Survivin and Ran were strongly expressed in GBM tissues, particularly in the perivasculature, and also in patient-derived GSC cultures. LLP-3 treatment disrupted the Survivin–Ran protein complex in cancer cells and abolished the growth of patient-derived GBM spheres in vitro and in vivo. This inhibition was dependent on caspase activity and associated with p53 status of cells. Immunohistochemistry showed that Survivin expression is significantly increased in recurrent GBM compared with newly diagnosed tumors, and temozolomide-resistant GBM spheres exhibited high sensitivities to LLP-3 treatment.

Conclusions: Disruption of the Survivin–Ran complex by LLP-3 abolishes survival and growth of GSCs both in vitro and in vivo, indicating an attractive novel therapeutic approach for GBM. Clin Cancer Res; 19(3); 631–42. ©2012 AACR.

Introduction

Glioblastoma multiforme (GBM), the most common primary tumor in the central nervous system (CNS), is an aggressive and highly lethal malignancy (1). Despite the use of surgical resection in conjunction with current adjuvant therapies, the median survival period of GBM patients is only 14.6 months (2). To overcome this devastating disease, it is crucial to deepen our understanding of the mechanisms that govern glioma cell survival and proliferation.

Within heterogeneous tumor cell populations, glioma-initiating cells, also known as glioma stem cells (GSC), constitute a subset of tumor cells that possess the ability to self-renew and give rise to diverse tumor cell types (3). Accumulating evidence has suggested that GSCs contribute to the development of therapy resistance by preferentially upregulating the DNA-damage checkpoint proteins (4). In response to DNA damage resulting from chemoradiotherapy, the checkpoint proteins are activated more efficiently in GSCs compared with non-GSCs, which subsequently suppress apoptosis by facilitating the DNA repair process following therapeutic insult. Survivin is an inhibitor of apoptosis (IAP) that forms heteromeric complexes with various proteins, including Aurora B, XIAP, and INCENP, and is among the top 5 tumor-specific genes in the human genome (5). Survivin is an attractive molecular target for novel cancer therapies because the disruption of Survivin signaling may impair vital processes in cancer cells, such as
Here, we show that Survivin and Ran are preferentially expressed by glioma stem cells (GSC) and targeting Survivin–Ran complex eradicates GSCs in glioblastoma multiforme (GBM). Patient-derived GSC cultures exhibited high expression of Survivin and Ran. A novel small molecule LLP-3, synthesized with an in silico structure-based design, disrupts the Survivin–Ran complex and eliminates the nucleocytoplasmic translocation of Ran effector molecule, TPX2, resulting in a mitotic defect of tumor cells. Treatment of GSCs with LLP-3 abrogates neurosphere formation in vitro and attenuates the growth of GSC-derived mouse intracranial tumors in vivo with a subsequent survival benefit for tumor-burdened mice. Temozolomide-resistant GBM cells derived from murine intracranial tumors exhibit a potent sensitivity to LLP-3. Immunohistochemistry with 75 patient samples reveals a statistically significant elevation of Survivin expression in recurrent GBM tumors following failure of temozolomide treatment and radiotherapy, which supports the significance of targeting the Survivin–Ran complex in GBM.

**Translational Relevance**

Here, we show that Survivin and Ran are preferentially expressed by glioma stem cells (GSC) and targeting Survivin–Ran complex eradicates GSCs in glioblastoma multiforme (GBM). Patient-derived GSC cultures exhibited high expression of Survivin and Ran. A novel small molecule LLP-3, synthesized with an in silico structure-based design, disrupts the Survivin–Ran complex and eliminates the nucleocytoplasmic translocation of Ran effector molecule, TPX2, resulting in a mitotic defect of tumor cells. Treatment of GSCs with LLP-3 abrogates neurosphere formation in vitro and attenuates the growth of GSC-derived mouse intracranial tumors in vivo with a subsequent survival benefit for tumor-burdened mice. Temozolomide-resistant GBM cells derived from murine intracranial tumors exhibit a potent sensitivity to LLP-3. Immunohistochemistry with 75 patient samples reveals a statistically significant elevation of Survivin expression in recurrent GBM tumors following failure of temozolomide treatment and radiotherapy, which supports the significance of targeting the Survivin–Ran complex in GBM.

mitosis, chromosomal assembly and segregation, and the inhibition of apoptosis (5–7). However, Survivin is not exclusively expressed by cancer cells. Actively dividing somatic cells in normal tissues prominently express Survivin, where it plays an essential role in their survival, proliferation, and tissue homeostasis (8). Therefore, anti-Survivin therapeutics could potentially damage normal cells, raising concerns about their feasibilities for clinical use. A better understanding of Survivin-associated pathways is crucial to the elucidation of tumor cell-specific Survivin function and the identification of key processes by which tumor cell division is dysregulated.

The small GTPase Ran is a regulator of bipolar mitotic spindle assembly – a critical step toward the ultimate segregation of chromatids into the 2 daughter cells during cell division (9). Depletion of Ran has been shown to profoundly impair spindle formation in HeLa cells, resulting in severe microtubule dysregulation and abnormal chromosome segregation (10). Recently, Xia and colleagues showed that the protein complex of Ran with Survivin promotes spindle formation in tumor cells (11). The formation of mitotic spindles is regulated by the Survivin–Ran complex facilitating the delivery of the Ran effector molecule, TPX2, to microtubules. Elevated expression of both Survivin and Ran were independently observed in various malignancies, including those of the prostate (12, 13), ovary (14, 15), breast (16, 17), and colon (18, 19) in addition to GBM (20). Interestingly, Ran silencing induced mitotic defects and cell death in tumor cells, while it did not impair mitosis or reduce the viabilities of normal cells (11). On the basis of these observations, we raised a hypothesis that, unlike normal cells, cancer cells depend on the interaction of Survivin and Ran for their survival, and that the disruption of the Survivin–Ran complex potentiates apoptotic death, particularly in tumor cells. In this study, we sought to address these questions.

**Materials and Methods**

**Ethics**

Experiments using human tissue-derived materials were carried out with the approval of the Institutional Review Board at the Ohio State University (Protocol Number: 2005C0075). All animal experimentation was conducted with the approval of the Institutional Animal Care and Use Committee at the Ohio State University (Protocol Number: 2009A0241).

**Cell cultures**

Two short-term sphere cultures were established from GBM specimens (GBM528 and 83) at the Ohio State University as described previously (21–24). Characteristics of 2 GBM sphere samples (GBM157 and 1600) and 1 nontumor human fetal brain-derived spheres (16wf) were also published previously (21–24). Detailed methods are described in the supplementary materials.

**Chemicals**

The Abbott8 compound was used as a probe ligand to obtain the most suitable conformation of Survivin dimer- accommodating ligand binding at the dimerization interface through the combined replica-exchange molecular dynamics and ensemble docking (25, 26). The Abbott8-bound Survivin conformation was then used to derive LLP-3 dimerization interface binder to interfere with the dimerization. LLP-3 is designed to add 2 benzyl groups to Abbott8 to displace Leu98 and Leu102 side-chain interactions from the other Survivin monomer.

**Fluorometric titration assay**

Fluorescence derived from LLP-3 was measured by Hitachi MPF-4000 spectrofluorimeter to assess the binding of LLP-3 to Survivin wild-type (WT) and mutant proteins, which were purified from Escherichia coli. Protein concentrations were determined on the basis of absorption at 280 nm.

**In situ proximity ligation assay**

To detect protein–protein interactions in situ, we used the DuoLink Proximity Ligation Assay (PLA) kit (Olink Bioscience) according to the manufacturer’s instructions. U87MG cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and incubated with a blocking solution for 30 minutes, followed by incubation with anti-Survivin and anti-Ran antibodies (1:500 anti-Survivin antibodies, Cat no. SC-17779; 1:100 anti-Ran antibodies, Cat no. SC-20802, both from Santa Cruz Biotechnology). Then, cells were incubated with the PLA probes for 1 hour at 37°C, followed by detection of the protein complex according to the manufacturer’s instructions.
Immunoprecipitation

Anti-Survivin antibody (Abcam; Cat no. ab24479) cross-linked with aminolink plus coupling gel using the Pierce Co-Immunoprecipitation kit (Thermo Fisher Scientific; Cat no. 26149) was incubated with 500 μg of cell lysates at 4°C. At 16 hours postincubation, protein was eluted from the gel and subjected to SDS gel electrophoresis using NuPage gel apparatus (Invitrogen) and immunoblotted for Ran and Survivin proteins using anti-Ran (Novus Biologicals; Cat no. NB500-237) and anti-Survivin antibodies.

Statistical Analysis

Values are given as mean ± SD, unless noted otherwise in the figure legend. The numbers of replicates are noted in the figure or legends. Comparison of mean values between multiple groups was evaluated by an ANOVA followed by Tukey test. Comparison of mean values between 2 groups was evaluated by the χ² or t tests. The Kaplan-Meier test was used to evaluate the survival analysis. For all statistical methods, a P value less than 0.05 was considered significant. All other materials and methods are described in Supplementary materials and in previously published literature (22–24, 27–29).

Results

Survivin and Ran are expressed in the perivascularature of GBM tissues

To investigate if Survivin and/or Ran are expressed by GSCs, we first carried out immunohistochemistry for Survivin and Ran with tissue sections that contain GBM tumors and adjacent normal tissues (Fig. 1 and Supplementary Fig. S1). Consistent with a recent study showing that GSCs are preferentially localized in the core of the tumor mass (30), both Survivin and Ran were predominantly expressed in the core lesions of GBM tissues relative to the peripheral lesions. Interestingly, we observed that Ran (+) cells preferentially
accumulate at the perivascular region within the core of the GBM tissues (Fig. 1), constituting 1 of the intratumoral lesions that indicate the presence of GSCs in vivo. Colocalization of Survivin and Ran with the GSC-associated proteins, CD133, SOX2, and MELK, was observed by immunohistochemistry (Fig. 1D). Conversely, normal brain tissues adjacent to tumors exhibited no or very low immunoreactivities to either of these proteins.

Survivin and Ran are highly expressed in GBM patient-derived GSCs

Next, we examined Survivin and Ran expression in the well-characterized patient-derived GSC cultures (22–24). Quantitative RT-PCR was used to compare the mRNA expression between undifferentiated GBM spheres (GSC cultures) and their sister cultures in prodifferentiation condition (non-GSC cultures). A phenotypic difference of these 2 sets of cells was primarily defined by the tumorigenicity. While undifferentiated GBM spheres were capable of forming GBM-like tumors in immunocompromised mouse brains, tumorigenic potentials were depleted in their sister cultures under the prodifferentiation condition (22–24). mRNA expression of both proteins was much higher in the 2 GSC samples compared with their non-GSC counterparts (GBM157 and GBM528; Fig. 2A and Supplementary Fig. S2A). We then carried out single-cell immunofluorescence by double-labeling Survivin- and Ran-expressing cells with cell-type-specific markers (Fig. 2B and Supplementary Fig. S2B). Survivin- and Ran-expressing cells were both positively immunoreactive to the undifferentiated neural cell markers Nestin, SOX2, and MELK, but were negative to the lineage-committed marker TuJ1. These data suggest that both Survivin and Ran are predominantly expressed by GSCs derived from GBM tissues.

Intermolecular interactions between LLP-3 and the Survivin dimerization interface

The antiapoptotic function of Survivin is mediated through both homodimerization and heterodimerization with various other proteins, such as caspase 3 and 7, XIAP, Ran, and Smac/DIABLO (11, 25, 31, 32). We, therefore, sought to identify a small molecule that interferes with the formation of Survivin complexes. A simulation model using the crystal structure of the Survivin protein revealed a unique singular region that either self-dimerizes or physically interacts with other protein partners (Fig. 3A, left). Specifically, Phe93 was predicted to be critical for Survivin protein–protein interactions (25). We then used the molecular structure of a small molecule, Abbott8, to design inhibitors of Survivin complex formation (Supplementary Fig. S3A). Abbott8, which was originally identified by a high-throughput screening nuclear magnetic resonance
The NMR experiment, binds to the Survivin protein near its dimerization interface (26). To design the Abbott8-derived Survivin inhibitors, we retained the core moiety of Abbott8, which occupies the hydrophobic binding pocket at the dimerization interface. We then designed and synthesized 11 analogs that are predicted to dock to the corresponding domain to affect its protein interaction (Supplementary Fig. S3B). We next tested the abilities of these small molecules to affect the in vitro growth of GSCs. We incubated GBM528 spheres with various doses of each compound and measured cell viabilities in each condition. Among the 11 compounds, LLP-2 and -3 exhibited the most potent inhibition of GSC viability (Fig. 3B). In particular, the structure of LLP-3 consisted of benzyl rings that are attached to the Abbott8 core moiety with ether links to displace Leu98 and Leu102 in the Survivin protein. A simulation of the binding energy for LLP-3/Survivin indicated that LLP-3 is a potent inhibitor of the Survivin protein. Collectively, we decided to focus on LLP-3 for further examination.

Characterization of LLP-3 as an inhibitor of Survivin–Ran complex formation

As a next step, we conducted a fluorometric titration assay to assess whether LLP-3 actually binds to the Survivin protein (Fig. 3C). Incubation of LLP-3 with the WT Survivin protein resulted in an increase in the LLP-3-derived fluorescence, while this result was not observed with incubation of LLP-3 with the SurvivinF101A/L102A protein harboring point mutations in the amino acid residues required for protein–protein interactions (33). Together, these data suggested that LLP-3 physically binds to the Survivin protein.

To determine the mechanism of action of the Survivin–LLP-3 complex, we examined its protein-binding partner(s) with a glioma cell line U87 and a sarcoma cell line HT1080. Specifically, we sought to identify the protein partner that fails to bind to Survivin at doses that are equivalent to or less than the IC50 for the GSC viability (Fig. 3B). Survivin is known to block apoptosis in cancer cells through physical interaction with the proapoptotic protein Smac/DIABLO; thus, we first investigated the effect of LLP-3 on the interaction of Survivin and Smac/DIABLO. We immobilized His-tagged Smac/DIABLO on copper-coated high-binding-capacity plates (Pierce) and incubated it with GST–GFP–Survivin in the presence of various concentrations of LLP-3. When we investigated the effect of LLP-3 on Survivin homodimerization or interaction of Smac/DIABLO with XIAP, another IAP with a strong homology to Survivin, we did not observe any inhibitory effects at concentrations of up to 200 μmol/L. Therefore, LLP-3 appeared to specifically bind to Survivin, but not to the other IAPs. Immunocytochemical analyses of LLP-3-treated cells exhibited consistent results. Analyses of α-tubulin staining showed that virtually all of the dividing cells exhibited mitotic defects, comprising multiple short mitotic spindles and abnormal DNA separation between daughter...
cells (Supplementary Fig. S4). Notably, LLP-3 did not alter the localization of Survivin at the kinetochores or midbody. These data suggest that LLP-3 does not affect the interaction of Survivin with other chromosomal passenger complex (CPC) proteins.

The small GTPase Ran seems to be involved in mitotic spindle assembly and the timing of cell-cycle transitions, at least partly through interaction with Survivin (34). Immunocytochemistry yielded findings that the cellular phenotype following LLP-3 treatment is similar to that caused by disruption of the Survivin–Ran interaction in HeLa cells (33). To test if LLP-3 blocks this interaction, immobilized His-tagged Survivin was incubated with GST-GFP–Ran in the presence of different concentrations of LLP-3. Abundance of bound protein was analyzed by Western blot (A) and ELISA (B) using anti-GFP antibody. C, visualization of Survivin–Ran interactions by *in situ* proximity ligation assay (PLA) in U87 cells. Cells were treated with either DMSO or 20 mmol/L of LLP-3 for 24 hours. Representative fluorescence images (left) exhibit PLA signal (red dots) and DAPI nuclear staining (blue) in U87 cells. Scale bars: 20 μm. Graph on the right indicates intensities of PLA signals of individual samples. For each sample, signals with at least 100 cells were measured. Error bars represent mean values ± SD; *P < 0.01*. D, immunocytochemical analysis of U87 cells with TPX2 and Survivin antibodies after treatment either DMSO or 40 mmol/L of LLP-3 for 24 hours. Representational images depicting the failure of TPX2’s colocalization with acetylated-α-tubulin in stable microtubule or midbody at telophase of LLP-3-treated U87 cells. Scale bars represent 20 μm (bottom). E, quantitative RT-PCR data depicting the changes in Survivin expression in U87MG (p53 WT line) and U87E6 (p53-inactivated line) after irradiation at the indicated doses. GAPDH was used as an internal control. Error bars represent the SD of 3 measurements. Asterisks (*) indicate statistical significance as determined by ANOVA (*P < 0.05*; left). A graph indicating the proportion of viable cells in U87MG or U87E6 treated with varying doses of LLP-3 for 72 hours. The IC50 of p53-inactivated cells was 13.6 μmol/L versus 38.1 μmol/L for p53 WT cells. Error bars represent the SD of 3 measurements. Asterisks (*) indicate statistical significance as determined by *t* test (*P*, 0.001; right).
LLP-3-induced inhibition of the Survivin–Ran interaction in U87 cells in situ (Fig. 4C). Fluorescence signals indicating the Survivin–Ran interaction were significantly decreased in LLP-3–treated cells compared with the control ($P < 0.01$). These data further confirmed the LLP-3–induced inhibition of the Survivin–Ran interaction in glioma cells. We then evaluated the expression of the Ran target protein TPX2 in LLP-3–treated U87 cells during cell division via immunocytochemistry (Fig. 4D). In untreated glioma cells, TPX2 was completely colocalized with acetylated α-tubulin (+) microtubules of the mitotic spindle at metaphase. In contrast, in LLP-3–treated glioma cells, TPX2 largely failed to be colocalized with the acetylated α-tubulin. At telophase, TPX2 immunoreactivity completely disappeared in LLP-3–treated cells (Fig. 4D). The same results were observed with LLP-3–treated HT1080 cells (Supplementary Fig. S4).

Next, we verified the effect of LLP-3 on cell cycle. Flow cytometry of propidium iodide (PI)-stained U87 cells exhibited that LLP-3 substantially reduced the proportions of cells in the S and G2–M phases (from 9% to 5% and from 25% to 17%, respectively) and increased G0–G1 cell population (from 60% to 74%; Supplementary Fig. S5A), suggesting that LLP-3 triggers cell-cycle arrest and subsequent tumor cell death. Similar data were obtained using HT1080 cells (Supplementary Fig. S5A). We then investigated whether LLP-3–induced cell death was actually caspase-dependent apoptosis. HT1080 cells were treated with LLP-3 in conjunction with z-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk), a pan-caspase inhibitor (26). LLP-3 treatment increased Annexin V-positive cells, suggesting that HT1080 cell death resulted from apoptosis. In combination of LLP-3 with zVAD-fmk, the proportion of Annexin V-positive cells was diminished to levels comparable with those in untreated cells (Supplementary Fig. S5B). These data suggest that LLP-3 triggers caspase-dependent apoptosis in tumor cells.

**Effects of LLP-3 are associated with p53 pathway**

One of the most commonly mutated or deleted genes in GBM is tumor suppressor p53, which signaling is altered in 87% of all GBM cases (35). To determine whether the LLP-3–mediated inhibition of the Survivin–Ran complex was p53-dependent, we used an isogenic glioma cell line U87 with a single difference in the p53 status — p53-intact U87MG and p53-inactivated U87E6 (35). Survivin expression following radiation treatment exhibited a striking difference between U87MG and U87E6 (Fig. 4E, left). After radiation, U87E6 cells, but not U87MG cells, exhibited markedly increased Survivin expression. Consistent with this expression data, the sensitivities of U87E6 cells to LLP-3 were significantly higher than those of U87MG cells (Fig. 4E, right). The IC_{50} for U87E6 and U87MG were 13.6 and 38.1 μmol/L, respectively. We then carried out the same investigation with mouse GSCs. For this experiment, mice with 2 distinct genetic backgrounds of p53^{+/−} and p53^{−/−} were used and mouse GSCs were enriched from tumors (Supplementary Fig. S6). Consistent with the above data using human glioma cell lines, p53 deletion was associated with elevated sensitivity to LLP-3. Collectively, these data suggested that the inhibitory effect of LLP-3 on the Survivin–Ran complex is associated with the p53 status of tumor cells.

**LLP-3 induces apoptotic cell death in organotypic slice cultures of GBM surgical specimens**

The organotypic slice cultures of GBM tissues enable us to determine the influence of drug treatment on tumor cells in situ without destroying the tumor microenvironment (28). Thus, we tested the effects of LLP-3 in this system (Supplementary Fig. S7). We carried out intratumoral injections of LLP-3, LLP-9 (a less potent compound), and dimethyl sulfoxide (DMSO; as a vehicle control). After a 16-hour incubation, the tumor slice cultures retained the histopathological characteristics of GBM (Supplementary Fig. S7, left). Immunohistochemistry of Ki-67 exhibited the presence of many proliferating tumor cells in the DMSO- and LLP-9–treated samples, but not in the LLP-3–treated samples. In turn, immunohistochemistry for caspase-3, a marker for apoptosis, exhibited abundant apoptotic cells in LLP-3–treated GBM tissues, but not in LLP-9- or DMSO-treated tissues. Collectively, LLP-3 treatment strongly suppressed GBM cell proliferation and induced apoptosis in tumor slice cultures.

**LLP-3–mediated inhibition of Survivin–Ran complex formation impairs growth of GSCs in vitro**

Next, we sought to determine whether the LLP-3–mediated disruption of the Survivin–Ran complex eliminates GSCs in tumors. Binding of Survivin and Ran in GBM83 sphere cells was confirmed with immunoprecipitation-Western blot (Fig. 5A). We then conducted a neurosphere formation assay — a surrogate assay to assess the effect on GSCs (Fig. 5B; ref. 21). Using 4 short-term cultures (GBM157, 83, 528, and 1600), we found that neurospheres formation is abrogated by LLP-3 treatment with an IC_{50} of 20 to 30 μmol/L in a dose-dependent manner. In contrast, normal neural progenitors from human fetal brain tissues (16wf) were less sensitive to LLP-3 treatment and exhibited an IC_{50} of 69.3 μmol/L. These data were not surprising, given that the expression of both Survivin and Ran is abundantly elevated in GSCs as opposed to normal progenitors (Fig. 2A).

The cell surface protein CD133 is a surrogate, if not a definitive or universal, marker for GSCs (36). We found that CD133 expression correlated with the presence of GSCs in a subset of our GBM samples (24). Using CD133 expression as an indicator of the presence of GSCs, we evaluated the effect of LLP-3 treatment on GBM157 cells (Fig. 5C). First, we tested whether LLP-3 changes the proportion of CD133-expressing GBM cells. In support of previous studies by several groups including ours, treatment of GBM157 with the first-line chemotherapy agent temozolomide (TMZ) or radiation at a dose equivalent to the IC_{50} for GSCs survival did not noticeably change the proportion of CD133-expressing cells. In contrast, LLP-3 significantly reduced the
proportion of CD133-positive cells, even at doses that are lower than the IC50 for both GSC sphere formation and survival (Figs 3B and 5C). Next, we separated GBM157 cells on the basis of CD133 expression and tested the effects of LLP-3 on individual fractions of GBM cells. The inhibitory effect of LLP-3 on the growth of CD133-positive cells is much more prominent compared with CD133-negative cells (Fig. 5B, right). Taken together, these results suggested that the LLP-3-mediated inhibition of the Survivin–Ran complex inhibits the growth of GSCs in vitro.

Figure 5. The effect of LLP-3 treatment on GSC growth in vitro and in vivo. A, lysed GBM83 sphere cells were subjected to communoprecipitation using anti-Survivin antibody and blotted for Ran and Survivin using anti-Ran and anti-Survivin antibodies. B, relative sphere cell numbers from 4 GBM samples (GBM528, GBM157, GBM83, and GBM1600) and normal fetal neurospheres (16wf) treated with varying doses of LLP-3. IC50 of 4 GBM samples GBM528, GBM157, GBM83, and GBM1600 were 34.25, 23.87, 29.13, 34.8 versus 69.37 μmol/L for normal fetal neurosphere, 16wf. C, histograms indicating the proportions of CD133-positive cells in GBM528 spheres treated with radiation, temozolomide, or LLP-3 for 48 hours (left). Relative numbers of CD133 (+) GSCs or CD133 (−) non-GSCs treated with LLP-3 (25 μmol/L) for 7 days. Error bars represent the SD of 3 measurements (right). D, orthotopic GBM tumors were established by intracranial xenografting of GBM83 spheres into immunocompromised mice (n = 8), which were subsequently treated with 25 mg/kg LLP-3 intraperitoneally (i.p.) 5 days a week for 2 weeks starting at day 10. Log-rank test was carried out on the Kaplan–Meier curves of the experimental animals. E, graph in top panel indicating the differences in tumor sizes between DMSO- and LLP-3-treated immunocompromised mice harboring intracranial tumors derived from GBM1600 spheres (n = 8). Two weeks after xenografting of GBM1600 cells into the mouse brains, a single dose of 2.5 pmol of LLP-3 or DMSO diluted with PBS was injected into tumor cavities. At 6 weeks postinjection, mice were sacrificed to evaluate tumor sizes by H&E staining intensities using the ImageJ software. Representative pictures of H&E staining of mouse tumors are shown on the right. Original magnification is ×10. Scale bar represents 100 μm. Asterisks (*) indicate statistical significance as determined by t test. Exact P value is indicated in the figure. Images at the bottom indicate TPX2 immunohistochemistry of both tumor groups. Original magnification is ×20. Scale bar represents 50 μm. The proportions of TPX2 (+) cells are indicated below; V, lateral ventricle; CC, corpus callosum.
LLP-3–mediated inhibition of Survivin–Ran complex formation impairs growth of GSC-derived intracranial murine tumors

Our in vitro results prompted us to investigate the in vivo effect of LLP-3 on tumor growth. To this end, we used the well-characterized GBM sphere-derived tumor models (GBM83 and 1600) (Fig. 5D and E; refs. 21, 24, 35). Both of these GBM sphere samples exhibited potent sensitivities to LLP-3 in vitro (Fig. 5B). With either no treatment or vehicle treatment, intracranial xenograft of GBM83 spheres created large GBM-like tumors in brains of immunocompromised mice with the median survival of 15.5 days (Fig. 5D). In contrast, LLP-3 treatment by intraperitoneal injection for 10 days (days 10–14 and days 17–21) significantly prolonged survival of tumor-burden mice and did not exhibit any lethality of mice until day 35 (Fig. 5D). When sacrificed at day 35, LLP-3–treated mice did not exhibit any obvious intracranial tumors (data not shown).

We then carried out pharmacodynamic analysis of LLP-3 to determine whether the effect is mediated through inhibition of the Survivin–Ran complex. To this end, we used the less aggressive GBM sphere sample (GBM1600), and carried out local injection of LLP-3 directly into their tumor cavities. Intratumoral LLP-3 or DMSO injection was carried out at day 14. We then assessed tumor sizes of both groups by H&E staining at 6 weeks postinjection. The overall tumor size was reduced by LLP-3 to approximately 50% (Fig. 5E, top). Immunohistochemistry showed that TPX2 expression was substantially decreased in LLP-3–treated tumors compared with the control tumors (9.1 ± 4.3% in the control group vs. 2.3 ± 0.8% in the LLP-3–treated group; Fig. 5D, bottom). Collectively, these data indicated that the LLP-3–mediated inhibition of the Survivin–Ran complex in GSCs results in diminished tumor growth in vivo.

Survivin expression is elevated in recurrent GBM tumors after therapeutic failure and is a prognostic indicator for postsurgical GBM patients

Recent studies have suggested that GSCs are more resistant to current chemoradiation therapies than non-GSCs (4). Thus, we predicted that if Survivin causally contributes to the development of therapy resistance in GSCs, then its expression should be elevated in tumors that recur after treatment failure. To address this question, we collected 75 GBM tissues, of which 44 were newly diagnosed surgical specimens and 31 were recurrent specimens, and compared the Survivin expression between these 2 groups via immunohistochemistry (Fig. 6A). For collection of recurrent tumors, we only included the patients who had undergone whole-brain radiation and TMZ chemotherapy. Immunohistochemistry yielded the strong correlation of Survivin expression with increased recurrence. Overall, recurrent tumors showed significantly higher levels of Survivin expression than newly diagnosed tumors (P < 0.05; Fig. 6A, right). To directly compare Survivin expression in matched newly diagnosed and recurrent GBM specimens, we collected paired GBM specimens from 10 cases. In further support of the earlier observations, immunohistochemical analyses showed an increase in Survivin expression in 8 out of 10 recurrent cases. Quantitative analyses of these samples showed significantly increased Survivin expression in correlation with therapeutic failure and subsequent recurrence (P < 0.05; Fig. 6A, right).

To assess the prognostic impact of Survivin protein expression, we further analyzed whether Survivin expression is predictive of postsurgical survival periods for GBM patients (Fig. 6B). Forty-two tumors were stained with Survivin antibody and divided into 2 groups on the basis of their immunostaining intensities. In agreement with a previous study (37), patients with higher proportions of Survivin-positive cells (more than 33% of tumor cells) in their GBM specimens exhibited shorter periods of both progression-free and overall survival. These data suggested that Survivin expression is predictive of postsurgical patient prognosis.

TMZ-resistant GBM cells are sensitive to LLP-3

In light of these clinical data, we sought to test whether TMZ-resistant GBM cells are sensitive to LLP-3 treatment. To this end, we obtained TMZ-treated intracranial murine tumors that were both derived from human GBM tumors (GBM12TMZ and GBM14TMZ) and the same patient-derived tumors without treatment (GBM12 and GBM14; ref. 38). Using these 4 samples, we established 2 paired GBM sphere cultures (Fig. 6C). In agreement with previous studies (40, 41), both GBM12TMZ and GBM14TMZ developed resistance to TMZ and yielded more sphere-forming GSCs in comparison with the parental GBM12 and GBM14 cells, respectively. These data indicated that TMZ treatment preferentially eradicates non-GSCs with less effects on GSCs. Analyses of these sphere cultures by quantitative RT-PCR showed that the expression of 2 ATP-binding cassette transporters, ABCB1 and ABCG2, were significantly higher in TMZ-resistant tumor cells (Supplementary Fig. S8). We then determined the sensitivities of these cultures to LLP-3 (Fig. 6C). The increase in sphere cell growth of GBM12TMZ relative to GBM12 was abolished following treatment with 25 μmol/L LLP-3. The same trend was also observed with the other pair of GBM cells; GBM14TMZ and GBM14. Collectively, these data suggested that LLP-3 potently inhibits the growth of TMZ-resistant GBM cells in vivo.

Discussion

Resistance to current therapies is a hallmark of GBM that distinguishes its clinical prognosis from those of other CNS tumors. The lethality of GBM results from the uncontrolled growth of recurrent tumors after therapeutic failure. In this study, we observed that recurrent GBMs expressed significantly higher Survivin than newly diagnosed, untreated GBMs. A recent study by Xia and colleagues showed that Survivin forms a protein complex with the small GTPase Ran in HeLa cells and its interaction supports microtubule stability and mitotic spindle assembly (11). Our data with the novel small-molecule LLP-3 supports the roles of Survivin in the Ran signaling in GSCs. Inhibition of the
Survivin–Ran interaction exerts a more potent effect on the in vitro growth of primary cultures pretreated with TMZ in vivo compared with nontreated cultures (Fig. 6C). Given that Survivin expression is elevated in TMZ-treated GBM xenografts relative to parental xenografts, therapy-resistant tumor cells, which accumulate in relapsed GBMs, are considerably more dependent on the Survivin–Ran complex for their survival.

GSCs can be enriched from surgical specimens of GBM using several cell surface antigens, such as CD133 (3), CD15 (39), L1CAM (40), and A2B5 (41). However, none of these proteins appears to be a universal GSC marker (42). In the current study, we focused on the well-characterized CD133-expressing GSCs (21–24) and used 2 assays to determine the effects of blocking Survivin–Ran complex formation on GSCs: the flow cytometric analysis of CD133 and an in vitro neurosphere formation assay. The data in Fig. 5 indicate that CD133-positive cells are more sensitive to LLP-3-induced cell death compared with CD133-negative cells. Sphere-forming potential of GBM cells was also significantly abolished by the LLP-3 treatment. However, taking the heterogeneity of GSCs into consideration, these data prevent us from drawing definitive conclusions with regard to the universal efficacy of the LLP-3 treatment on GSCs. Furthermore, we cannot rule out the possibility that LLP-3 exerts its effects on additional molecular targets, such as Survivin-binding partners and/or some unknown molecules outside of the context of Survivin action. Further studies are required to fully elucidate the roles of the Survivin–Ran complex and LLP-3 in controlling the growth of GSCs and other cancer cells.

The regulation of apoptosis in response to cellular stress or DNA-damaging agents is balanced between the actions of proapoptotic molecules, such as p53, caspases, and SMAC/DIABLO and antiapoptotic molecules, such as Survivin and XIAP. Survivin is upregulated in various cancers after irradiation and the loss of p53 function is a common phenomenon in many cancers. We found that radiation increases Survivin expression in p53-inactivated glioma cells but not in p53-intact isogenic glioma cells (Fig. 4). Thus, p53 is likely an essential gatekeeper gene required for the activity of Survivin in GBM. We also observed the increased Survivin expression in recurrent GBM tissues compared with nontreated tumors and Survivin expression is predictive of postsurgical patient survival. A, representative pictures of Survivin immunohistochemistry analyses of newly diagnosed (nontreated) and recurrent GBM tissues. Original magnification of the top is ×20 and the bottom is ×40. Scale bars in top and bottom panels are 50 and 20 μm, respectively. Quantified immunostaining results with newly diagnosed and recurrent GBM tissues in unmatched cases (n = 75), and primary and recurrent tumors in 10 matched GBM cases. The average of the proportion of Survivin (+) cells in each sample set is represented by the red horizontal bar (P < 0.05; right). B, a Kaplan–Meier curve depicting the survival of GBM patients with high and low levels of Survivin expression. Exact P value is indicated in the figure. C, graphs indicating the relative cell numbers of 4 GBM sphere samples (GBM12, GBM12TMZ, GBM14, and GBM14TMZ) in the presence of LLP-3 (25 μmol/L) or DMSO following 7 days of treatment.

Figure 6. Recurrent GBM tissues exhibit higher Survivin expression than nontreated tumors and Survivin expression is predictive of postsurgical patient survival. A, representative pictures of Survivin immunohistochemistry analyses of newly diagnosed (nontreated) and recurrent GBM tissues. Original magnification of the top is ×20 and the bottom is ×40. Scale bars in top and bottom panels are 50 and 20 μm, respectively. Quantified immunostaining results with newly diagnosed and recurrent GBM tissues in unmatched cases (n = 75), and primary and recurrent tumors in 10 matched GBM cases. The average of the proportion of Survivin (+) cells in each sample set is represented by the red horizontal bar (P < 0.05; right). B, a Kaplan–Meier curve depicting the survival of GBM patients with high and low levels of Survivin expression. Exact P value is indicated in the figure. C, graphs indicating the relative cell numbers of 4 GBM sphere samples (GBM12, GBM12TMZ, GBM14, and GBM14TMZ) in the presence of LLP-3 (25 μmol/L) or DMSO following 7 days of treatment.

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spheres with LLP-3 selectively inhibited the growth of GSCs the small GTPase Ran. Treatment of patient-derived GBM LLP-3, using a structure-based computational drug design. patients. We developed a novel small-molecule inhibitor, nostic value for predicting postsurgical survival of GBM uncover of the roles of the Survivin–Ran complex in cancers. normal cells. Further investigation will be required to stream targets cooperate to function as cancer genes, leading to actions specifically, if not exclusively, regulate mitotic pro-
cessing in GSCs has not been determined. However, it is intriguing to postulate that Survivin, Ran, and their down-
stream targets cooperate to function as cancer genes, leading to their differential expression in cancer cells compared with normal cells. Further investigation will be required to uncover of the roles of the Survivin–Ran complex in cancers.

In this study, we provide the first evidence that both Survivin and Ran are expressed abundantly by GSCs in GBM. The Survivin protein is substantially upregulated in therapy-relased GBM tumors and its expression has prog-
nostic value for predicting postsurgical survival of GBM patients. We developed a novel small-molecule inhibitor, LLP-3, using a structure-based computational drug design. LLP-3 effectively inhibited the interaction of Survivin with the small GTPase Ran. Treatment of patient-derived GBM spheres with LLP-3 selectively inhibited the growth of GSCs both in vitro and in vivo. These inhibitory effects of LLP-3 were dependent on the p53-status of the tumor cells. These data indicate that Survivin–Ran-directed therapeutics may constitute a novel class of targeted agents for future GBM treatment strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: H. Guvenc, M.S. Pavlyukov, I. Nakano Development of methodology: H. Guvenc, M.S. Pavlyukov, H. Kurt, C-H. Kwon, D. Bhasin, J.N. Sarkaria, C. Li Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Guvenc, M.S. Pavlyukov, Y.K. Banasavadi-Siddigrewa, P. Mao, R. Yamada, C-H. Kwon, D. Bhasin, S. Chettiar, G.J. Kitange, J.N. Sarkaria Analysis and interpretation of data (e.g., statistical analysis, bio-
statistics, computational analysis): H. Guvenc, M.S. Pavlyukov, K. Joshi, Y.K. Banasavadi-Siddgrewa, P. Mao, D. Bhasin, I.H. Park, C. Li Writing, review, and/or revision of the manuscript: H. Guvenc, M.S. Pavlyukov, K. Joshi, C-H. Kwon, J.N. Sarkaria, C. Li, I. Nakano Administrative, technical, or material support (i.e., reporting or organ-

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Impairment of Glioma Stem Cell Survival and Growth by a Novel Inhibitor for Survivin–Ran Protein Complex

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