Highly penetrative, drug-loaded nanocarriers improve treatment of glioblastoma

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Current therapy for glioblastoma multiforme is insufficient, with nearly universal recurrence. Available drug therapies are unsuccessful because they fail to penetrate through the region of the brain containing tumor cells and they fail to kill the cells most responsible for tumor development and therapy resistance, brain cancer stem cells (BCSCs). To address these challenges, we combined two major advances in technology: (i) brain-penetrating polymeric nanoparticles that can be loaded with drugs and are optimized for intracranial convection-enhanced delivery and (ii) repurposed compounds, previously used in Food and Drug Administration-approved products, which were identified through library screening to target BCSCs. Using fluorescence imaging and positron emission tomography, we demonstrate that brain-penetrating nanoparticles can be delivered to large intracranial volumes in both rats and pigs. We identified several agents (from Food and Drug Administration-approved products) that potently inhibit proliferation and self-renewal of BCSCs. When loaded into brain-penetrating nanoparticles and administered by convection-enhanced delivery, one of these agents, dithiazanine iodide, significantly increased survival in rats bearing BCSC-derived xenografts. This unique approach to controlled delivery in the brain should have a significant impact on treatment of glioblastoma multiforme and suggests previously undescribed routes for drug and gene delivery to treat other diseases of the central nervous system.

Of the ∼40,000 people diagnosed with primary brain tumors in the United States each year, an estimated 15,000 have glioblastoma multiforme (GBM), a World Health Organization grade IV malignant glioma (1). Despite considerable research efforts, the prognosis for GBM remains poor: median survival with standard-of-care therapy (surgery, systemic chemotherapy with temozolomide, and radiation) is 14.6 mo (2) and 5-y survival is 9.8% (3), with the vast majority of GBMs recurring within 2 cm of the original tumor focus (4). Histopathologically, GBM is characterized by its infiltrative nature and cellular heterogeneity, leading to a number of challenges that must be overcome by any presumptive therapy.

The blood–brain barrier (BBB) is a major obstacle to treating GBM (5). It is estimated that over 98% of small-molecule drugs and ~100% of large-molecule drugs or genes do not cross the BBB (6). Delivery of chemotherapeutics to the brain can be potentially achieved by using nanocarriers engineered for receptor-mediated transport across the BBB (7, 8), but the percentage of i.v. administered particles that enter the brain is low. It is not yet clear whether sufficient quantities of drug can be delivered by systemically administered nanoparticles to make this a useful method for treating tumors in the human brain. An alternate approach is to bypass the BBB: Clinical trials have demonstrated that the BBB can be bypassed with direct, locoregional delivery of therapeutic agents. For example, local implantation of a drug-loaded biodegradable polymer wafer (presently marketed as Gliadel), which slowly releases carmustine over a prolonged period, is a safe method for treating GBM. However, use of the Gliadel wafer results in only modest improvements in patient survival, typically 2 mo (9, 10). In prior work we showed that these wafers produce high interstitial drug concentrations in the tissue near the implant, but—because drugs move from the implant into the tissue by diffusion—penetration into tissue is limited to ~1 mm, which could limit their efficacy (11, 12).

We hypothesize that treatment of GBM can be improved by attention to three challenges: (i) enhancing the depth of penetration of locally delivered therapeutic agents, (ii) providing for long-term release of active agents, and (iii) delivering agents that are known to be effective against the cells that are most important in tumor recurrence. The first challenge can be addressed by convection-enhanced delivery (CED), in which agents are infused into the brain under a positive pressure gradient, creating bulk fluid movement in the brain interstitium (13). Recent clinical trials show that CED is safe and feasible (14–16), but CED alone is not sufficient to improve GBM treatment. For example, CED of a targeted toxin in aqueous suspension failed to show survival advantages over Gliadel wafers (14, 17). Although CED of drugs in solution results in increased penetration, most drugs have short half-lives in the brain and, as a result, they disappear soon after the infusion stops (17, 18).

Loading of agents into nanocarriers—such as liposomes, micelles, dendrimers, or nanoparticles—can protect them from clearance. Significant progress has been made in CED of liposomes to the brain (19), although it is not clear that liposomes offer the advantage of long-term release. By contrast, CED of polymeric nanoparticles, such as nanoparticles made of poly(lactide-coglycolide) (PLGA), offers the possibility of controlled agent release. However, CED of PLGA nanoparticles, which are typically 100–200 nm in diameter, has been limited by the failure of particles to move by convection through the brain interstitial spaces (20–23), which are 38–64 nm in normal brain (24) and 7–100 nm in regions with tumor (25).

To overcome the first and second challenges, it is necessary to synthesize polymer nanocarriers that are much smaller than conventional particles and still capable of efficient drug loading and controlled release. We report here reliable methods for making PLGA nanoparticles with these characteristics.

Drug developers have long been frustrated by the BBB, which severely limits the types of agents that can be tested for activity in the brain. We reasoned that creation of safe, versatile, brain-penetrating nanocarriers should enable direct testing of novel agents that address the complexity of GBM biology. For example, cells isolated from distinct regions of a given GBM bear grossly different expression signatures but seem to arise from a common


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Isolated using this protocol were 74 μm particles for long-term storage. However, lyophilization can also cause nanoparticles to aggregate, making them difficult to resuspend in an aqueous solution. Furthermore, particle aggregation, if it did occur, could complicate CED infusion and restrict penetration in the brain. To reduce aggregation, we used the disaccharide trehalose as an excipient, which was added to the nanoparticle suspension at a ratio of 0.5:1 (trehalose:nanoparticles) by mass immediately before lyophilization (46, 47).

The addition of trehalose did not alter nanoparticle size, morphology, or yield. SEM images demonstrated that trehalose enhanced the separation of nanoparticles from one another (Fig. 1E) compared with nanoparticles lyophilized without trehalose (Fig. 1D). Reconstitution of cryoprotected nanoparticles resulted in a homogenous solution, whereas reconstitution of nanoparticles lyophilized without trehalose cryoprotection resulted in sedimentation over time, which caused clogging of the CED device and prevented infusion at a consistent pressure. We also determined hydrodynamic diameters of nanoparticles in PBS solution by Zetasizer (Malvern) and observed a similar trend in size change when the modifications described above were applied (Table S1).

Results

Synthesis of PLGA Nanoparticles. PLGA nanoparticles were synthesized using a single-emulsion, solvent evaporation technique. Dichloromethane (DCM) was chosen initially as the solvent owing to its ability to dissolve a wide range of hydrophobic drugs. To produce particles of the desired diameter, we developed a partial centrifugation technique. Specifically, after solvent evaporation and before particle washing, the particle solution was subjected to low-speed centrifugation (8,000 × g for 10 min), which caused larger particles to pellet while keeping the smaller particles in the supernatant. The initial pellet contained comparatively large nanoparticles and was removed (Fig. 1A). Nanoparticles in the supernatant were collected and washed using high-speed centrifugation (100,000 × g for 30 min). SEM showed that nanoparticles isolated using this protocol were 74 ± 18 nm in diameter and morphologically spherical (Fig. 1B). The typical yield for this fabrication was 12 ± 2%. In comparison, nanoparticles made using the same materials but with conventional centrifugation techniques were 150 ± 30 nm in diameter, with an average yield of 55 ± 5% (Fig. 1C). Organic solvents used for preparing polymer solution are known to affect the size of PLGA nanoparticles synthesized through emulsion procedures (45). In particular, partially water-miscible organic solvents—such as benzyl alcohol, butyl lactate, and ethyl acetate (EA)—allow nanoparticle formulation through an emulsion-diffusion mechanism and are able to produce smaller nanoparticles than water-immiscible solvents such as DCM (45). Therefore, we hypothesized that replacing DCM with partially water-miscible organic solvents could improve the yield of small nanoparticles. EA was chosen because of its low toxicity. Nanoparticles synthesized using EA as the solvent instead of DCM were 65 ± 16 nm in diameter and morphologically spherical. The yield was improved with EA: 44 ± 3% (Fig. 1D).

Cryptopretreatment to Prevent Aggregation of PLGA Nanoparticles. Lyophilization is a technique commonly used to stabilize nanoparticles for long-term storage. However, lyophilization can also prevent infusion at a consistent pressure. We also determined hydrodynamic diameters of nanoparticles in PBS solution by Zetasizer (Malvern) and observed a similar trend in size change when the modifications described above were applied (Table S1).

CED of PLGA Nanoparticles in the Rat Brain. We next sought to assess the effects of particle size and cryoprotection on intracranial CED and volume of distribution (Vd). We synthesized both small PLGA nanoparticles and standard PLGA nanoparticles. Before lyophilization, nanoparticles from each group were further divided into two groups: with or without trehalose cryoprotection. Nanoparticles were loaded with coumarin-6 (C6), a fluorescent dye commonly used for visualization. Small and standard nanoparticles had mean diameters of 71 nm ± 13 nm and 147 nm ± 27 nm, respectively. Consistent with previous work (48), release of C6 from nanoparticles was negligible (<0.5%) at 72 h.

Sixteen nude rats received 20-μL infusions (Vi) of C6-loaded nanoparticles into the right striatum via CED (n = 4 per group). Animals were killed 30 min after infusion and their brains were sectioned and analyzed using fluorescence microscopy to determine Vd. Both small size and trehalose cryoprotection independently contributed to increased penetrance of nanoparticles in brain parenchyma, and small nanoparticles with cryoprotectant resulted in the best distribution in the brain (Fig. 2). We call the small, trehalose-treated carriers brain-penetrating nanoparticles, because of this enhanced ability to distribute in the brain via CED. Mean Vd for brain-penetrating particles was 74 ± 7 mm3 (Vd/Vi = 3.7 ± 0.3), whereas mean Vd for standard particles without trehalose was 11 ± 3 mm3 (Vd/Vi = 0.6 ± 0.1). Subgroup analyses demonstrate that both small size and coating with trehalose independently improve Vd (P < 0.05). Interestingly, for brain-penetrating nanoparticles compared with standard nanoparticles, the Vd/Vi increased toward the theoretical limit of 5, which is usually only achievable by ideal free drugs in solution (23, 49–51).

Live, Noninvasive Imaging of Brain-Penetrating Nanoparticles in the Rat Brain Using PET. The clinical translation of delivery systems for the treatment of intracranial diseases has been hindered by an
ability to noninvasively characterize in vivo distribution. We applied a modular radiolabeling strategy to permit noninvasive, quantitative PET imaging of our brain-penetrating nanoparticles (S2). PLGA nanoparticles were modified to display surface-bound palmitoyl-arginine, which enabled easy radiolabeling of nanoparticles with N-(4-[(18)F]fluorobenzyl)propanamido-PEG2-Biotin ([18F]NBP4), a biotinylated, gamma-emitting compound that can be detected with PET (53, 54). [18F]NBP4-labeled and C6-loaded PLGA nanoparticles were synthesized and delivered via CED to the right striatum of five Sprague Dawley rats. Three rats received infusions of brain-penetrating PLGA nanoparticles, and the other two rats received infusions of standard nanoparticles without trehalose ($V_d = 20 \mu L$ for both groups) (Fig. 3A). When measured noninvasively and quantitatively with PET imaging, the mean $V_d$ for the brain-penetrating nanoparticles was $111 \pm 3$ mm$^3$ ($V_d/V_i = 5.5 \pm 0.2$), whereas the mean $V_d$ for the standard nanoparticles was $53 \pm 23$ mm$^3$ ($V_d/V_i = 2.6 \pm 1.2$) (Fig. 3B). Postmortem analysis using fluorescence microscopy revealed that the mean $V_d$ for the brain-penetrating nanoparticles was $82 \pm 5$ mm$^3$ ($V_d/V_i = 4.1 \pm 0.2$), whereas the mean $V_d$ for the standard nanoparticles was $11 \pm 4$ mm$^3$ ($V_d/V_i = 0.5 \pm 0.2$) (Fig. 3C). The $V_d/V_i$ determined for these brain-penetrating nanoparticles by fluorescence analysis ($4.1 \pm 0.2$) was similar to the $V_d/V_i$ obtained in particles without [18F]NBP4 coatings ($3.7 \pm 0.3$; see above), suggesting that our method for [18F] labeling does not hinder particle penetration. Thus, consistent with imaging results from destructive fluorescence microscopy of brain sections, quantitative analysis of noninvasive PET imaging demonstrated that brain-penetrating nanoparticles reached a larger volume of spatial distribution than standard nanoparticles (Fig. 3D). We note that the differences between distribution volumes calculated from PET images and fluorescence images are likely due to the substantial differences in sensitivity and spatial resolution for these two imaging techniques.

**CED of Brain-Penetrating Nanoparticles in the Pig Brain.** Rodent brains are much smaller than human brains, so it is difficult to assess whether the $V_d$ obtained after CED in the rat is relevant to treatment of human disease. To extend our analysis to larger brains, we infused brain-penetrating, C6-loaded PLGA nanoparticles into the striatum of pig brains ($n = 4$) using our CED technique ($V_i = 338 \mu L$). Animals were killed 120 min postinfusion and their brains were analyzed with fluorescence microscopy to determine $V_d$ (Fig. 4). Brain-penetrating PLGA nanoparticles delivered by CED penetrated pig brain tissue with a mean $V_d$ of $1,180 \pm 37$ mm$^3$, which resulted in $V_d/V_i = 3.5 \pm 0.1$, similar to the value obtained in the rat (again, much closer to the theoretical limit of 5 than previously achieved with similar nanoparticles). The extent of nanoparticle penetration in the pig brain was $>1$ cm (Fig. 4). Our brain-penetrating nanoparticles distribute to volumes that are clinically relevant, because the vast majority of GBMs recur within 2 cm of their original location (4). Even greater penetration is possible in humans, because infusions volumes of up to 72 mL have been used safely in previous clinical trials (14).

**Delivery of Chemotherapy for Solid Brain Tumor.** We next sought to assess whether these brain-penetrating PLGA nanoparticles could be used to treat intracranial tumors. For initial studies, we created intracranial tumors in immunocompromised rats by injection of U87MG, the most commonly used human GBM cell line, and we treated the animals with CED of paclitaxel, a drug previously shown to inhibit proliferation of U87MG. Our results showed that paclitaxel was efficiently encapsulated into brain-penetrating nanoparticles, and CED of paclitaxel-loaded brain-penetrating nanoparticles enhanced survival in tumor-bearing rats: Median survival times for rats receiving brain-penetrating, paclitaxel-loaded particles were significantly longer than for rats receiving either free paclitaxel or standard, paclitaxel-loaded nanoparticles (SI Text and Fig. S1).

We are aware that a histopathologic hallmark of GBM is its infiltrative nature. The U87MG cell line has been propagated in cell culture for many years and has lost its infiltrative nature in vivo. After intracranial injection, U87MG cells form solid tumors that are histopathologically distinct from human GBM (55) (Fig. S2A). In contrast, several recent studies have demonstrated that a murine xenograft model using human BCSCs has the ability to recapitulate human GBM histopathology (37, 56). To test whether BCSCs were able to form such tumors in nude rats, we inoculated GS5, a well-characterized BCSC line (37, 57) in rat brains. Consistent with the findings in mouse brains (37, 57), GS5 tumors in the brain of nude rats are highly infiltrative and histopathologically similar to human GBM (Fig. S2B and C). Therefore, we sought to improve the translational relevance of our studies by (i) identifying agents that are effective against patient-derived BCSCs and (ii) delivering these agents in an animal model that is reflective of human GBM.

We screened a library of ~2,000 compounds that have been used by humans, in FDA-approved products, against GS5 for growth-inhibitory activity (Fig. 5A) (58, 59). Briefly, GS5 cells were plated in 96-well format, treated with 5 μM drug, and evaluated for viability 3 d later using the thiazolyl blue tetrazolium bromide (MTT) assay. Initial hits were subsequently evaluated for inhibition of GS5 sphere formation, a measure of BCSC self-renewal. Thirty-two candidate compounds were identified (Table
S2), some of which were later confirmed in an independent high-throughput screen in BCSCs (60). The BCSC growth-inhibiting activity of many compounds was confirmed using AlamarBlue. One compound in particular, the anti-helminthic cyanine dye dithizone iodide (DI), potently inhibited GS5 proliferation, with an IC50 of 79 nM. Treatment with DI inhibited GS5 sphere formation, a measure of BCSC self-renewal, by 94%. DI-mediated inhibition of BCSC sphere formation was further confirmed by a limiting dilution assay with pretreatment of DI (Fig. S3). Additionally, DI decreased the CD133+ cell population by 57% (Fig. 5B). DI was evaluated in two additional BCSC lines isolated from the same GBM patient (PS11 and PS16), and showed similar anti-BCSC effects (Fig. 5B and Fig. S3).

We next evaluated whether CED of brain-penetrating, DI-loaded nanoparticles could prevent tumor growth in our histopathologically relevant model of GBM. DI was loaded into brain-penetrating nanoparticles with encapsulation efficiency of 19% and yield of 18%. Brain-penetrating, DI-loaded nanoparticles were spherical and had an average diameter of 70 ± 19 nm (Fig. 6 A and B). DI was released from brain-penetrating nanoparticles in a controlled manner over several weeks (Fig. 6C). To evaluate their efficacy in vivo, brain-penetrating, DI-loaded nanoparticles were administrated via a single infusion into rat brains bearing GS5-derived tumors. Brain-penetrating DI nanoparticles significantly increased the median survival of tumor-bearing rats (Fig. 6D). Kaplan–Meier analysis revealed that rats treated with brain-penetrating, DI-loaded nanoparticles had significant improvements in median survival, which was over 280 d. By contrast, rats receiving standard nanoparticles, free drug, blank/unloaded nanoparticles, and no treatment had a median survival of 180, 177, 156, and 147 d, respectively (P < 0.005 for each comparison) (Fig. 6D). We also note that in our experience to date, 48 control animals (i.e., animals receiving GS5-derived tumors but no treatment) all died of histologically confirmed tumors within 200 d of tumor initiation, suggesting that the probability of tumor initiation using our procedures is near 100%.

We also tested two other compounds that exhibited activity in our in vitro screening experiments. Both anisomycin and digoxin performed well on in vitro assays against BCSCs and were loaded efficiently into brain-penetrating nanoparticles that provided controlled release. In pilot experiments, however, CED delivery of anisomycin-loaded or digoxin-loaded particles provided no survival benefit to rats with intracranial BCSC-derived tumors (Figs. S4 and S5).

**Discussion**

In this report, we describe a unique strategy for the treatment of GBM that addresses the two most important obstacles to effective therapy: (i) the infiltrative nature of GBM and (ii) the genetic heterogeneity of the tumor and chemoresistance of BCSCs, which give rise to drug delivery and discovery challenges, respectively. To overcome the challenges associated with drug delivery, we developed a controlled-release delivery system composed of brain-penetrating PLGA nanoparticles that can penetrate to substantially (approximately sevenfold) higher volumes than conventional PLGA nanoparticles when delivered intracranially using CED. The penetration of these particles is as good as any previously reported nanoparticle systems; For example, the Vd/Vt achieved in our studies is comparable to those achieved with nanoposomosal delivery systems in rats (61). PLGA particles have many advantages over liposomal formulations including lower toxicity and control of drug release. Further, we showed that the brain-penetrating ability of these particles extends to large animals: PLGA nanoparticles delivered in pig brains using CED penetrated to volumes of \(~1,180 \text{ mm}^3\). Because the vast majority of GBMs recur within 2 cm of the original tumor focus (4), the penetrative capacity of these brain-penetrating nanoparticles when delivered by CED can address the infiltrative nature of GBM. We also surface-modified nanoparticles with \([^{18}F]NPB4\) using streptavidin–biotin conjugation, which allowed us to track the nanoparticles during the CED procedure using noninvasive PET imaging. This capability will allow clinicians to visualize nanoparticles delivered by CED and ensure distribution of the therapeutic agent throughout the brain regions most likely in need of treatment.

In comparison with currently available nanocarrier drug delivery systems, this platform has at least three clear advantages. First, the polymer has an excellent safety profile: PLGA was part of an FDA-approved formulation in 1969 and has been safely used in clinical practice since that time. Specifically, PLGA is commonly used in suture material and is a component of several controlled-release drug delivery products. Second, the release kinetics of PLGA nanoparticles can be more easily modulated than those of competing nanocarrier systems used in intracranial applications, namely liposomes and micelles. Third, the versatile surface modification approach described in this study enables rapid, modular attachment of biotinylated agents, thereby allowing for efficient labeling of nanoparticles with a host of cell-targeting and -penetrating agents. Finally, the exceptionally small diameters allow these nanoparticles to penetrate relatively large, clinically relevant volumes when delivered by CED. In short, this is a versatile delivery platform for the central nervous system, which we have optimized for translational medicine.

This delivery platform allows for the direct testing of new agents for treating GBM. BCSC resistance to conventional chemotherapeutics is a major challenge in GBM. We used a library screening approach to identify agents that have improved activity against BCSCs. Of the 1,937 compounds we screened, we settled on DI for initial testing owing to its abilities to inhibit growth, inhibit self-renewal, and encourage differentiation of cells it fails to kill. We note that recent reports by other groups confirm many of the other potential drugs we identified, such as emetine (60). Brain-penetrating, DI-loaded PLGA nanoparticles inhibit growth of intracranial tumors in an animal model that closely reflects many aspects of human GBM. We demonstrated the effectiveness...
of our approach on intracranial tumors that were initiated in the striatum, which is the most common experimental site. Although this is not the most common location for occurrence of human GBM, which is a disease of the white matter, the striatum is the largest, most homogenous area available in the rodent brain and is thus the best location for evaluating the effectiveness of CED. In addition, our experimental results in the rodent and pig demonstrate that the CED-delivered nanoparticles are able to travel through white matter tracts and across the corpus callosum, which enhances their clinical relevance. More broadly, our work suggests that improved treatment of GBM might be achievable if obstacles pertaining to both the infiltrative and chemoresistant properties of the disease can be sufficiently overcome.

We have tested our platform with two other drugs that seemed highly active against BCSCs in our screen: digoxin and anisomycin. Interestingly, efficacy in vivo—as determined by increase in median survival after a single treatment by CED with drug-loaded, brain-penetrating nanoparticles—was not predictable from its potency in cell culture. For example, the cardiac glycoside digoxin and the antibiotic anisomycin exhibited IC_{50} values against BCSCs comparable to DI. Although these drugs were loaded efficiently into brain-penetrating nanoparticles, and administered without problem to rats with intracranial tumors, the survival benefits were modest compared with DI. Further work may reveal conditions that lead to prolonged survival with CED of brain-penetrating nanoparticles loaded with digoxin or anisomycin—by refinements in V_{ct} nanoparticle design, or dosing scheme—but these results highlight the striking effectiveness of DI-loaded, brain-penetrating nanoparticles when administered by CED. The observation that DI is more effective in vivo than other drugs with comparable in vitro activity also suggests that it will be important to evaluate the effectiveness of these agents in intracranial tumors formed from BCSC representing different subgroups in The Cancer Genome Atlas (62)—which likely differ in vascularity, BBB integrity, and other factors—to identify the relationship between tumor biology, agent activity, and distribution of nanoparticles in the complex cellular milieu of brain tumors.

Although brain-penetrating PLGA nanoparticles were evaluated here against intracranial tumors with small molecule drugs, the system can be tailored for application to a host of CNS diseases. For example, surface modification or size fractionation could produce particles well suited for the treatment of certain neurodegenerative disorders, such as Parkinson disease or Huntington disease, as well as diseases with localized cerebral dysfunction, such as stroke. These particles also have the potential to encapsulate not only hydrophobic drugs but also a variety of nucleic acids for gene therapy applications (63). The particles are simple in composition, which should aid in clinical translation. A recent study reported by Hanes and coworkers (64) suggests that a dense PEG coating is needed to allow nanoparticles as large as 114 nm in diameter to diffuse passively in the brain: Without the PEG coating, transport of particles of this size was strongly hindered. Our results suggest that PEG is not needed for unhindered transport of smaller particles (~70 nm) during CED. Owing to their ability to penetrate brain tissue, their construction from safe components, and their ability to control agent release, we anticipate that this brain-penetrating PLGA nanoparticle delivery platform will have significant clinical impact.

**Methods**

**Nanoparticle Synthesis.** Nanoparticles loaded with C6 or paclitaxel were synthesized by a single-emulsion solvent evaporation technique. One hundred milligrams of PLGA (50:50; Polysciences and Birmingham) and agents to be encapsulated were dissolved in 2 mL DCM or EA. The polymer/drug solution was then added dropwise to 4 mL of 2.5% polyvinyl alcohol (PVA) as the outer aqueous phase and sonicated to form an emulsion. The emulsion was poured into a beaker containing aqueous 0.3% (vol/vol) PVA and stirred at room temperature for 3 h (DCM as solvent) or 5 h (EA as solvent) to allow the solvent to evaporate and particles to harden.

To synthesize standard nanoparticles, following the solvent evaporation phase, the nanoparticle solution was subjected to typical centrifugation speeds (11,500 × g for 15 min, three times) and the pellet was collected. To synthesize small nanoparticles, following the solvent evaporation phase, the nanoparticle solution was centrifuged at low speed (8,000 × g for 10 min) to pellet the large nanoparticles. The supernatant was decanted and small nanoparticles were collected through high-speed ultracentrifugation (100,000 × g for 30 min, two times).

To prevent nanoparticle aggregation during lyophilization, trehalose was added to the final aqueous solution at a ratio of 0.5:1 (trehalose:nanoparticles) by mass immediately before lyophilization.

**Drug Screening.** Drug screening was performed in clear 96-well plates using a compound library that contains 1,937 compounds that are or were components of an FDA-approved product (58, 59). The procedure for screening is depicted in Fig. 5A. Cell proliferation and sphere formation assays were performed as described below.

**Antitumor Activity in Xenograft Model.** To establish tumors for evaluation of paclitaxel-loaded PLGA nanoparticles, nude rats were first anesthetized with a ketamine/xylazine mixture (n = 5 per control group, n = 10 per treatment group). Animals were then prepped with betadine and alcohol and placed in a stereotactic frame. A linear midline incision was made and a 1.5-mm-diameter hole was drilled in the skull 3 mm lateral and 0.5 mm anterior to the right side of the brain. The burr hole was drilled using a mechanical drill and then the skull was removed using a burr drill. The rats were then anesthetized, prepped, and placed in a stereotactic frame. The wound was reopened and the Hamilton syringe was oriented as described previously. Twenty microliters of either nanoparticles (100 mg/mL) or equivalent free drug were infused continuously at a rate of 0.667 μL/min. Following infusion, the syringe was left in place for 5 min, after which it was removed. The drug solution was then added to the cage with free access to food and water mixed with ibuprofen. Seven days after tumor inoculation, all rats received a single treatment as indicated. Rats were again anesthetized, prepped, and placed in a stereotactic frame. The wound was reopened and the Hamilton syringe was oriented as described previously. Twenty microliters of either nanoparticles (100 mg/mL) or equivalent free drug were infused continuously at a rate of 0.667 μL/min. Following infusion, the syringe was left in place for 5 min, after which it was removed. The drug solution was then added to the cage with free access to food and water mixed with ibuprofen. Seven days after tumor inoculation, all rats received a single treatment as indicated. Rats were again anesthetized, prepped, and placed in a stereotactic frame. The wound was reopened and the Hamilton syringe was oriented as described previously. 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The drug solution was then added to the cage with free access to food and water mixed with ibuprofen.
Statistical Analysis. All data were collected in triplicate, unless otherwise noted, and reported as means and SD. Comparison of two conditions was evaluated by a paired Student t test. Kaplan–Meier analysis was used to evaluate the effect of various treatments on survival. A P ≤ 0.05 was considered to indicate a statistically significant difference.

More information is given in SI Text.

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SI Text
Poly(lactide-co-glycolide) (PLGA) nanoparticles loaded with paclitaxel were synthesized by two techniques: brain-penetrating and standard paclitaxel-loaded nanoparticles were spherical and of expected diameters (75 ± 20 nm and 159 ± 38 nm, respectively) (Fig. S1A). All nanoparticle fabrications (brain-penetrating and standard) were loaded with paclitaxel, having encapsulation efficiencies of ~60% and yields of >35%. Controlled-release experiments showed that brain-penetrating and standard PLGA nanoparticles released paclitaxel similarly, with ~75% of the encapsulated drug released from each formulation over the first 28 d of incubation (Fig. S1B). Both brain-penetrating and standard paclitaxel nanoparticles inhibited growth of U87MG in vitro, exhibiting lower IC_{50} values (39 nM and 37 nM, respectively) than free drug (169 nM). None of the blank nanoparticle formulations exhibited cytotoxicity (Fig. S1C).

To determine in vivo efficacy, we generated U87MG-derived xenografts in the right striatum of nude rats. Tumor-bearing rats were divided into five groups that received either no treatment (n = 5); convection-enhanced delivery (CED) of brain-penetrating, paclitaxel-loaded nanoparticles (n = 10); CED of standard, paclitaxel-loaded nanoparticles (n = 10); CED of blank (unloaded), brain-penetrating nanoparticles (n = 5); or CED of paclitaxel in solution (n = 10). Consistent with our previous experience, rats tolerated all procedures well; no perioperative toxicity was observed in any of the treatment groups. Kaplan–Meier analysis revealed that rats treated with brain-penetrating, paclitaxel-loaded nanoparticles had significant improvements in median survival (46 d) compared with all other groups (38 d for standard nanoparticles, 30 d for free drug, 31 d for blank/unloaded nanoparticles, and 27 d for no treatment; P < 0.05 for each comparison) (Fig. S1D).

SI Materials and Methods

Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Cell Culture. Human glioma cell line U87MG was purchased from American Type Culture Collection. Cells were grown in a 37 °C incubator containing 5% CO₂ and cultured in DMEM (In-vitrogen) supplemented with 10% FBS (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

Brain Cancer Stem Cell Cultures from Human Glioblastoma Multiforme Tissue. All studies were approved by the Yale University Institutional Review Boards. Tumor samples classified as glioblastoma multiforme (GBM) based on World Health Organization criteria were obtained from neurosurgical patients at Yale–New Haven Hospital who had provided informed consent. Within 1–3 h of surgical removal, tumors were washed, cut into <1-mm³ fragments, and enzymatically dissociated into single cells. Digested fragments were filtered using a 70-μm cell strainer (BD Falcon) and collected in culture medium. The G55 cell line was kindly provided by the Lamszus laboratory (1). All brain cancer stem cell cultures (BCSCs) were collected and cultured in Neurobasal A medium (Invitrogen) supplemented with B27 (Invitrogen), fibroblast growth factor-2 (20 ng/mL; Peprotech), and epidermal growth factor (20 ng/mL; Peprotech). Growth factors were added at least weekly.

SEM. Particle size was characterized by SEM. Samples were mounted on carbon tape and sputter-coated under vacuum with gold in an argon atmosphere using a Dynavac Mini Coater set at 40 mA current (Dynavac). SEM was carried out using a Philips XL30 SEM and a LaB₆ electron gun with an accelerating voltage of 3 kV. Mean particle diameters and size distributions were determined by image analysis of ~200 particles using ImageJ (National Institutes of Health). The same images were used to qualitatively assess particle morphology.

Characterization of Nanoparticle Loading. To determine the loading and encapsulation efficiency of coumarin-6 (C6) nanoparticles, 3–5 mg of nanoparticles were dissolved in 1 mL of DMSO at room temperature. Loading of C6 in the nanoparticles was quantified based on the solution’s fluorescence intensity (excitation 444 nm and emission 538 nm) using a spectrophotometer (SpectroMax M5; Molecular Devices). Blank nanoparticles were used for background control. Paclitaxel loading was quantified using HPLC as previously reported (2). The same approach was used to characterize loading of dithiazanine iodide (DI) in nanoparticles, except that the concentration of DI was determined based on its absorbance at 655 nm.

In Vitro Controlled Release. Nanoparticles (3–5 mg) were suspended in 1 mL of PBS (pH 7.4) and incubated at 37 °C with gentle shaking (70 rpm). Release of C6, paclitaxel, or DI was monitored at several time points over a 4-wk period. At each sampling time, the nanoparticle suspension was centrifuged for 15 min at 21,000 × g. The supernatant was removed for quantification of C6, paclitaxel, or DI and replaced with an equivalent volume of PBS for continued monitoring of release. Detection of C6, paclitaxel, or DI was conducted using the methods described above.

Fluorescence-Based Imaging of Nanoparticle Distribution in Rat Brain. All procedures involving animals were approved by the Yale University Institutional Animal Care and Utilization Committee (IACUC). Female athymic (Ncr-nu/nu) nude rats were maintained in a sterile environment. Rats were anesthetized with ketamine/xylazine solution via i.p. injection and given analgesic. The scalp was prepped with betadine and alcohol. The rat was then placed in a stereotactic head frame. A midline incision was made and a 1.5-mm-diameter hole was drilled in the skull 3 mm lateral and 0.5 mm anterior to the bregma. The right striatum was targeted. A 26G Hamilton syringe, with 28G stepdown inner cannula, was inserted to a depth of 5 mm. The tissue was allowed to equilibrate mechanically for 5 min. Subsequently, 20 μL of nanoparticles (100 mg nanoparticles/mL solution) (n = 4/group) was infused (V_t) continuously at a rate of 0.667 μL/min. Following infusion, the syringe was left in place for 5 min to allow for equilibration. For distribution studies, animals were killed 30 min postinfusion; the brains were harvested and frozen.

Nanoparticle distribution was quantified using previously described methods (3). Specifically, each brain was serially sectioned into 150-μm slices on a cryostat. The distribution of nanoparticles in the slices was captured on a fluorescence stereoscope (Lumar V12; Carl Zeiss) using a Cy3 filter. The exposure time was optimized to achieve maximum dynamic range at the infusion site while simultaneously avoiding saturation. Exposure time for each nanoparticle group was individually optimized, to adjust for differences in loading between nanoparticle groups. Within each group of nanoparticles, the exposure time was held constant. The distribution volume (V_d) of the nanoparticles was calculated using a custom Matlab 7.2 (MathWorks) script, which generated a binary image from the grayscale images and calculated the area of particle penetration. The threshold for the binary operation was...
10% of the maximum fluorescent intensity. The total \( V_a \) was calculated by multiplying the distribution area in each slice by the slice thickness (150 µm) and summing the volumes of all slices.

**Synthesis of \(^{18}\text{F}\)fluoro benzylpropanamido-PEG\(_2\)-Biotin Nanoparticles.** \(^{18}\text{F}\)fluoro benzylpropanamido-PEG\(_2\)-Biotin ([\(^{18}\text{F}\)]NPB4) was prepared as previously described (4), with 28 ± 14% radiochemical yield, >98% radiochemical purity, and 1–2 mCi/nmol specific activity. In preliminary experiments, \([^{18}\text{F}]\)NPB4 was conjugated to avidin surface-modified PLGA nanoparticles by incubating 7 mg of nanoparticles with -0.6 mCi of \([^{18}\text{F}]\)NPB4 for 1 h at room temperature. When this solution was centrifuged to pellet the nanoparticles, less than 1% of the total added radioactivity was detected in the wash. We estimated that <1% of available avidin sites on the nanoparticles were occupied by the \([^{18}\text{F}]\)NPB4. Each rat received a total dose of 100–300 µCi.

**PET-Based Imaging of Nanoparticle Distribution in Rat Brain.** For noninvasive imaging studies, Sprague Dawley rats (\( n = 5 \)) were anesthetized with ketamine/xylazine and a 26G guide cannula was placed in the skull (Plastics One) to enable nanoparticle infusions while data collection was ongoing. The right striatum was targeted. The guide cannula was secured to the surface of the skull with dental cement (Henry Schein) and surgical screws. Once in the scanner, rats were maintained on isofluorane anesthesia [2% (vol/vol)], and an infusion needle was threaded through the cannula to the target brain region. Emission data were collected during the infusion and for 30 min after completion with a Focus 220 small animal PET scanner (Siemens Medical Solutions). A transmission scan (\(^{60}\text{Co} \) source, 9 min) was collected before the emission scan. Rats were killed immediately after the scan and frozen in liquid nitrogen for later tissue sectioning and fluorescence microscopy. PET data were binned into 0.5- to 10-min frames and reconstructed with the ordered subset expectation maximization algorithm, with corrections for attenuation, decay, randoms, and scatter. The resulting pixel size was 0.949 × 0.949 × 0.796 mm, with an effective image resolution of ∼1.5 mm. Radial concentration profiles were extracted from each data frame and thresholded to 10% of the maximum value to determine the spatial volume of distribution.

**Fluorescence-Based Imaging of Nanoparticle Distribution in Pig Brain.** Nanoparticle infusions were performed in the striatum of Yorkshire pigs (\( n = 4 \)) to evaluate \( V_a \) in a large animal model. Pigs were anesthetized with ketamine/xylazine, intubated, and maintained with isofluorane/oxygen/NO\(_2\). The head was positioned such that the horizontal zero plane passed through bregma and was parallel to a line between the upper margin of the infraorbital ridge and the upper margin of the external auditory meatus. The scalp was prepped with betadine and alcohol. A linear midline incision was made and a 1.5-mm-diameter hole was drilled in the skull 11 mm lateral to bregma. A 26G Hamilton syringe, with 28G stepdown inner cannula, was inserted to a depth of 28 mm. The tissue was allowed to equilibrate mechanically for 5 min. Subsequently, 337.5 µL of nanoparticle solution were continuously infused at a rate of 0.5 µL/min for 30 min, 0.75 µL/min for 30 min, and 1 µL/min for 300 min. Following infusion, the syringe was left in place for 120 min, after which it was removed. Animals were subsequently killed; the brains were harvested, frozen, and sectioned as described above. Nanoparticle distribution was quantified using the methods described above. Exposure time was held constant between all animals.

**Cell Proliferation Assays.** For primary screening, a slightly modified thiazol blue tetrazolium bromide (MTT) assay was used to quantify the effects of drugs on cell proliferation. Briefly, cells were cultured in 96-well plates (Falcon). Three (for BCSC studies) or six (for U87MG studies) days after treatment, medium was removed and replaced with fresh medium containing 10% MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) solution (4.14 mg/mL). Four hours after incubation at 37 °C, all of the media was removed. Formazan was dissolved in DMSO and the OD was measured at 590 nm. The relative inhibition on growth was determined using the following formula: Growth inhibition = (control OD – sample OD) / control OD.

Proliferation was also assessed and IC\(_{50}\) calculated using AlamarBlue (Invitrogen) fluorescence. Briefly, cells were plated at subconfluent concentrations in black clear-bottomed 96-well plates (Falcon) with drug concentrations spanning eight orders of magnitude. Three or six days postplating (as above), AlamarBlue was added at the manufacturer’s recommended concentration. Cells were plated at 37 °C for 200 min and quantified (excitation 544 nm and emission 590 nm). Fluorescence measurements were corrected for background media and drug fluorescence and normalized to the mean of vehicle measurements. IC\(_{50}\) values were determined using four-parameter logistic modeling using normalized point estimates.

**Sphere Formation Assay.** BCSCs were plated as single-cell suspensions of five cells per microliter in 48-well plates (Falcon). Cells were treated with 1 µM drug or equivalent concentration of DMSO. Growth factor was supplemented on day 5. Wells were counted on day 7. Colonies containing more than five cells were considered to be spheres. Percent inhibition was calculated as (Control number of spheres – sample number of spheres)/control number of spheres.

**Flow Cytometry.** BCSCs were plated as single-cell suspensions in six-well plates with 100 nM drug or DMSO. Three days after plating, suspensions were collected and flow cytometry performed. Briefly, following reconstitution in 0.5% BSA in PBS (wt/vol), dissociated cells were washed in cold PBS and subsequently incubated with biotin-conjugated anti-CD133(PE) antibody (Miltenyi Biosciences). Suspensions were incubated with avidin-conjugated AlexaFluor 488 (Invitrogen) and read on a FACScan flow cytometer (BD Biosciences). Geometric means were calculated in FlowJo (TreeStar, Inc.), corrected for background (secondary only), and normalized to DMSO-only treated cells.

Fig. S1. Synthesis and antitumor effects of paclitaxel-loaded nanoparticles on U87MG cell culture and intracranial tumors in the rat. (A) Morphology of standard (Left) and small (Right) nanoparticles used in studies. (Scale bar, 200 nm.) (B) Controlled release of paclitaxel from nanoparticles. (C) Effects of free paclitaxel and paclitaxel-loaded nanoparticles on U87MG cell proliferation, in vitro. (D) Kaplan–Meier survival curves for tumor-bearing rats with indicated treatments: blue line, brain-penetrating paclitaxel nanoparticles (NPs) (median survival 46 d); red line, standard paclitaxel NPs (median survival 38 d); green line, free paclitaxel (median survival 30 d); yellow line, blank NPs (median survival 31 d); gray line, no treatment (median survival 27 d). Rats treated with brain-penetrating, paclitaxel-loaded NPs had statistically significant improvements in median survival compared with all other groups ($P < 0.05$ for each comparison).

Fig. S2. H&E staining of tumors derived from U87MG cells and BCSCs. (A) Implantation of U87MG results in solid tumor formation (arrow) in the rat brain. (B–E) Implantation of glioma stem cells (GSC5) into the striatum of a nude rat results in diffuse tumor formation. H&E staining demonstrates a highly cellular, infiltrative tumor (asterisk) with invasion of the corpus callosum (arrows) and spread to the contralateral hemisphere. Numerous mitotic figures (+) are noted within a single high-power field. These histopathologic findings are highly similar to those found in human GBM.
Fig. S3. Inhibition of BCSC self-renewal by DI, as determined by in vitro limiting dilution assays. To assess the self-renewal capacity of these cells when treated with DI, we used a pretreatment strategy as reported by Diamandis et al. (1) but with the addition of a third cell plating density. Pretreatment was used to ensure that what we were observing was not simply cell killing. Briefly, cells were grown in T125 flasks under previously described conditions. Either DI or vehicle solvent DMSO was added to the flask to a final concentration of 100 nM. In accordance with Diamandis et al. (1), cells were further incubated for 1 wk. At the end of 1 wk, the surviving cells were centrifuged and resuspended in non-drug-containing medium. Following TrypanBlue viability assessment, cells were aliquoted and diluted to the following concentrations: one cell per microliter, one cell per 10 μL, and one cell per 100 μL. Three separate 96-well plates were used, one for each concentration. One hundred microliters of each suspension was added to each well of a corresponding 96-well plate. Therefore, 96 wells were evaluated for each set of experimental conditions; in sum, >3,000 wells were evaluated. Growth factor was supplemented on day 4 of the experiment. For counting, plates were retrieved from the incubator on day 7 and evaluated under 4× magnification. The total number of neurospheres was

Legend continued on following page
noted for each well. For our purposes, “neurosphere” was defined as a tight group of more than eight cells. The total number of neurospheres for each plate was calculated. Raw neurosphere counts were normalized to the most densely-plated vehicle-treated group. Despite cell line differences in neurosphere formation, a decrease in self-renewal by ~50% was noted following DI pretreatment at all plating densities. The experiment was repeated with similar findings noted.

Fig. S4. Evaluation of anisomycin on BCSCs in vitro and in vivo. (A–C) In vitro evaluation of anisomycin on BCSCs. (A) Anisomycin treatment inhibited BCSC proliferation. Cell proliferation was determined using the standard MTT assay described in the main text. (B) Treatment with anisomycin at 1 or 5 μM inhibited BCSC sphere formation. Detailed methods for the sphere formation assay are found in the main text, with an inoculation of 500 cells. Of note, the BCSC line PS11 was used in this evaluation. (C) Treatment with anisomycin at 1 μM decreased the CD133+ population in the BCSC line PS11, as determined by flow cytometry. (D–F) Characteristics, including (D) morphology, (E) size distribution, and (F) controlled-release profile of brain-penetrating NPs loading with anisomycin. (G) Kaplan–Meier survival curves for tumor-bearing rats with indicated treatments: blue line, control NPs (n = 6); red line, brain-penetrating anisomycin NPs (n = 6).
Fig. S5. Evaluation of digoxin on BCSCs in vitro and in vivo. (A–C) In vitro evaluation of digoxin on BCSCs. (A) Digoxin treatment inhibited BCSC proliferation. (B) Treatment with digoxin at 1 or 5 μM inhibited BCSC sphere formation. Of note, the BCSC line PS11 was used in this evaluation. (C) Treatment with digoxin at 1 μM decreased the CD133+ population in the BCSC line PS11, as determined by flow cytometry. (D–F) Characteristics, including (D) morphology, (E) size distribution, and (F) controlled-release profile of brain-penetrating NPs loaded with digoxin. (G) Kaplan–Meier survival curves for tumor-bearing rats with indicated treatments: black line, no treatment (n = 6); red line, control NPs (n = 6); green line, brain-penetrating digoxin NPs (n = 6).
Table S1. Mean hydrodynamic diameter of nanoparticles of different formulations in PBS measured by Zetasizer

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Size before lyophilization, nm</th>
<th>Cryoprotectant</th>
<th>Size, nm</th>
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<tr>
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<td>165.3</td>
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<td>Trehalose</td>
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<td>DCM large</td>
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Nanoparticles were synthesized using two different solvents, ethyl acetate (EA) and dichloromethane (DCM). Standard-sized and small nanoparticles were fabricated as described previously. Trehalose was added as a cryoprotectant, as described previously. Nanoparticle size was measured before and after lyophilization. To measure the size of nanoparticles following lyophilization, the nanoparticles were resuspended in PBS and subjected to measurement after brief sonication using a water bath sonicator (2510; Branson).

Fig. S6. Replicate of experiment described in Fig. 6. Kaplan–Meier survival analysis of tumor-bearing rats with indicated treatments: blue line, DI brain-penetrating NPs (median survival >250 d); gray line, no treatment (median survival 113 d); yellow line, blank NPs (median survival 115.5 d).
<table>
<thead>
<tr>
<th>No.</th>
<th>Compound name</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;, μM</th>
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