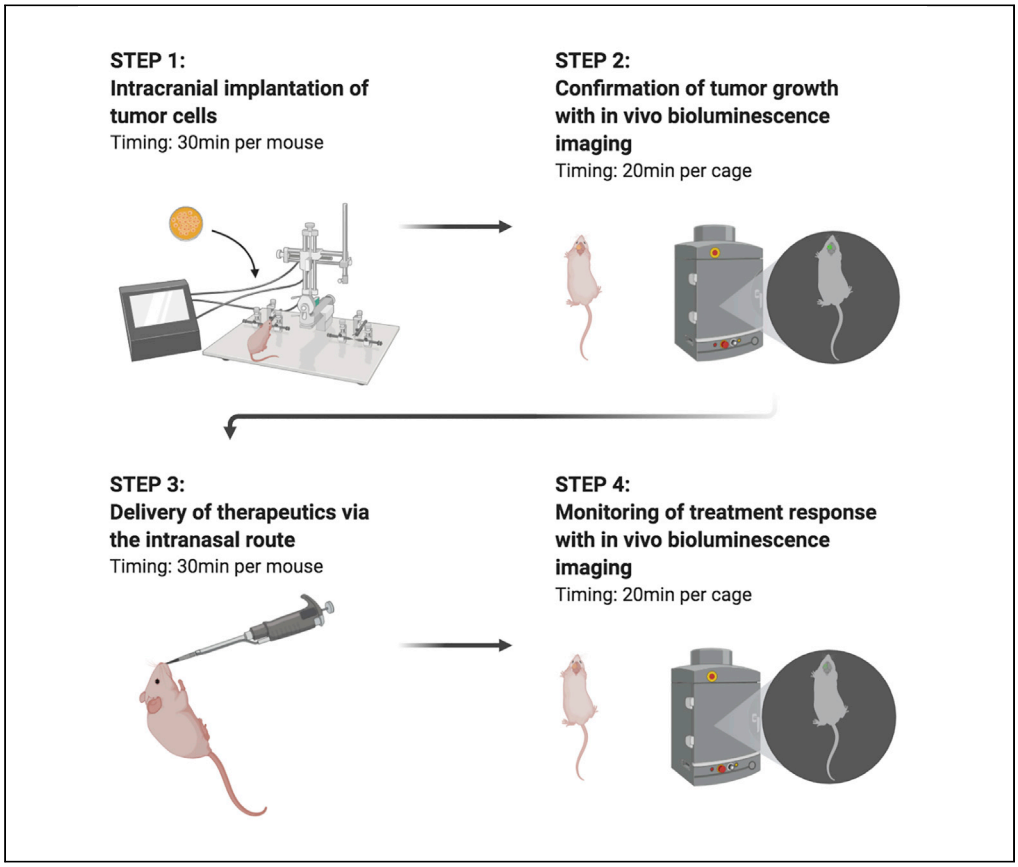
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Intranasal delivery of experimental compounds in orthotopic brain tumor mouse models



Effective therapeutics for malignant primary brain tumors, such as glioblastomas (GBMs), are urgently needed. To facilitate and expedite early-phase GBM therapeutic development, we describe a protocol that allows the intranasal delivery of experimental compounds in GBM orthotopic mouse models. Compounds delivered through this route can bypass the blood-brain barrier and thus help validate effective therapeutic targets for GBMs.

Katharina M. Eyme, Litia Carvalho, Christian E. Badr

badr.christian@mgh.harvard.edu

HIGHLIGHTS

Using patient-derived tumor cells to study and image GBM tumor growth in mice

Delivery of therapeutics via the intranasal route to bypass the blood-brain barrier

This delivery method can expedite early-phase testing of GBM therapeutics

Eyme et al., STAR Protocols 2, 100290
March 19, 2021
https://doi.org/10.1016/j.xpro.2020.100290

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Intranasal delivery of experimental compounds in orthotopic brain tumor mouse models

Katharina M. Eyme,[1,4] Litia Carvalho,[1,2,3] and Christian E. Badr[1,2,3,5,*]

[1]Department of Neurology, Massachusetts General Hospital, Boston, MA 02129, USA

[2]Neuroscience Program, Harvard Medical School, Boston, MA, USA

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: badr.christian@mgh.harvard.edu
https://doi.org/10.1016/j.xpro.2020.100290

SUMMARY

Effective therapeutics for malignant primary brain tumors, such as glioblastomas (GBMs), are urgently needed. To facilitate and expedite early-phase GBM therapeutic development, we describe a protocol that allows the intranasal delivery of experimental compounds in GBM orthotopic mouse models. Compounds delivered through this route can bypass the blood-brain barrier and thus help validate effective therapeutic targets for GBMs.

For complete details on the use and execution of this protocol, please refer to Pinkham et al. (2019).

BEFORE YOU BEGIN

Generation of luciferase expressing GBM cells

Timing: 5 days

Luciferase reporters are commonly used for monitoring tumor growth in small animals and for estimating tumor burden following intracranial implantation of GBM cells in mice. To stably express Firefly luciferase (Fluc) in primary GBM cells, we use a lentivirus vector containing Fluc and a fluorescent protein such as mCherry, separated by an internal ribosomal entry site (IRES) element and cloned into a lentivirus vector under the control of the strong constitutive cytomegalovirus (CMV) promoter to produce a LV-Fluc-mCherry. We have successfully used such lentivirus system to stably express, and simultaneously detect bioluminescence and fluorescence in several patient-derived GBM cells.

Transduction of GBM cells with LV-Fluc-mCherry:

1. Plate one million cells in one well of a 6-well plate.
2. After 24 h, add LV-Fluc-mCherry lentivirus vector at a multiplicity of infection (MOI) of 10–50 in the presence of 8 μg/mL polybrene.
3. After 16–24 h, spin down the cells at 1,500 × *g* for 5 min and wash with PBS and add fresh culture medium then incubate the cells for at least 48 h prior to testing Fluc expression and/or detecting mCherry fluorescence.
4. Determine the transduction efficiency by analysis of the mCherry expression using fluorescence microscopy.
5. For detection of Fluc expression, its substrate D-luciferin (450 μM) is added either directly to the cells, in a 96 well optical plate. Alternatively (for more sensitive detection) the substrate could be

added to the cell lysate. The signal is measured for 10 s to acquire photon counts and integrated over 2 s using a luminometer.

6. Following a successful transduction of GBM cells with LV-Fluc-mCherry, both Fluc and mCherry genes will integrate within the genome and therefore the cells will stably express the reporter. At this point the cells can be expanded and either directly used for intracranial implantation or cryopreserved for later use.

⚠ CRITICAL: Lentiviruses are considered a biological hazard and therefore all experiments using such system should be performed in biosafety level 2 certified laboratories.

Pause point: Fluc-expressing cells can be frozen and stored for future use prior to the next step.

Preparing GBM cells for implantation in mice

Timing: 1–4 weeks

Typically, patient-derived primary GBM cells are thawed and expanded 1–4 weeks prior to the planned in vivo experiment. We usually thaw one vial of frozen cells containing 1×10^6 cells which will be cultured in a 60 mm cell culture dish. It is best to prevent long-term expansion of primary GBM cells. Therefore, it is possible to thaw multiple frozen vials for experiments with a large animal cohort that require high number of cells. Short-term cell culture will minimize genetic and metabolic adaptability of tumor cells to an artificial environment (rich in glucose and O_2, controlled pH, etc.). This is also important in order to decrease the risk of mycoplasma contamination (cells to be implanted are confirmed to be mycoplasma negative few days prior to tumor implantation).

Primary GBM cells maintained under serum-free conditions typically grow as neurospheres (spheroids). These cells are cultured in DMEM/F12 or in Neurobasal medium (NBM), which is commonly used as a culture medium in the field (Lee et al., 2006). For our protocol, DMEM/F12 is supplemented with: B27 supplement (without vitamin A; final concentration 1×), recombinant human EGF (final concentration 20 ng/mL), recombinant human FGF2 (final concentration 10 ng/mL) and heparin (final concentration 2 μg/mL). Cells are passaged once a week and cell culture medium is changed once a week, in addition fresh cell culture medium is added three times a week.

Reagent	Final concentration	Amount
DMEM/F12 (4°C)	–	49 mL
B27 supplement (−20°C)	1×	1 mL
Recombinant human EGF (−20°C)	20 ng/mL	10 μL
Recombinant human FGF2 (−20°C)	10 ng/mL	10 μL
Heparin (4°C)	2 μg/mL	5 μL

The supplemented cell culture medium is stored at 4°C for up to 7 days.

One week before implantation: check cells

Timing: 1 h

Expand and count cells, make sure cells look healthy, confirm expression of Firefly luciferase (Fluc).

7. Count cells
   a. Spin down cells at 1,500 × *g* for 5 min and remove the supernatant.
   b. Dissociate the neurospheres using 100 μL accutase and let them incubate for 1 min at 37°C.
   c. Add 5 mL of plain DMEM medium to wash cells, spin them down at 1,500 × *g* for 5 min once again, then remove the supernatant.
   d. Resuspend cells in 1 mL of cell culture medium.

e. Count cells.
   i. The number of cells needed for implantation varies depending on the cell line used. We typically implant 10,000 cells/mouse for fast-growing and highly aggressive tumor cells, and 50,000 cells/mouse for all other GBM cells.

Note: Trypan blue (1:1) can be used to facilitate counting and verify viability and health of the cultured cells. Make sure to correct for dilution when calculating the number of cells.

8. Confirm stable luciferase expression
   a. Confirm that cells express Fluc by measuring Fluc activity. As previously outlined, we typically use a lentivirus system to stably co-express Fluc and a fluorescent reporter. In this case, the expression of the fluorescence reporter (and indirectly luciferase expression) can be confirmed using a fluorescence microscope without the need to disturb or lyse the cells.

⚠ CRITICAL: The health of GBM cells is critical for seeding after intracranial implantation and for tumor growth. If you do not have enough cells, the cells do not look healthy, or the luciferase is not stably expressed, consider postponing the experiment. In case the cells test positive for mycoplasma, you should consider thawing a new batch of cells whenever possible or, alternatively, treat the cells for mycoplasma removal (abm Mycoplasma Elimination Cocktail, 0.05 µL/mL of cell culture medium) for several days prior to their injection in mice. In case the cells have an altered morphology, are growing slower than usual, or are unable to form tight spheres, you should discard them and thaw a new vial. If Fluc expression is low, or in case not all GBM cells were stably transduced with the lentivirus expressing Fluc, consider re-infecting the cells or sorting them based on high expression of the fluorescence reporter (which is co-expressed with Fluc).

⏸ Pause point: Cells can be frozen and stored. However, the prior step will have to be repeated before the protocol can be continued.

One day before implantation: check and prepare cells

⏱ Timing: 20 min

9. Count cells to ensure that the desired cell count (1.5 times the number of mice to be implanted multiplied with the amount of cells to implant per mouse, e.g., for 40 mice and 50,000 cells/mouse requires 1.5 × 40 × 50,000 cells) is obtained before continuing with the IC injections the next day.
   a. Spin down cells at 1,500 × *g* for 5 min and remove the supernatant.
   b. Dissociate the neurospheres using accutase and let them incubate for 1 min at 37°C in a water bath.
   c. Add 5 mL of plain medium to wash cells, spin them down once again at 1,500 × *g* for 5 min, and remove the supernatant.
   d. Resuspend cells in 1 mL of cell culture medium.
   e. Count cells
      i. The number of cells needed for implantation varies depending on the cell line used. We typically implant 10,000 cells/mouse for fast-growing and highly aggressive tumor cells, and 50,000 cells/mouse for all other GBM cells.
10. Split cells into two or more cell culture dishes depending on how many aliquots of cells you will need the next day. This will minimize handling of cultured cells in a given plate and therefore the risk of contamination while collecting aliquots of cells for implantation the next day.

We typically plate enough cells to implant 12–15 mice during a time period of 3 h. After 3 h, the remaining cells are discarded, and a new batch of cells is collected for subsequent surgeries (as detailed below).

Note: The size of the plate should be selected according to the number of cells to be plated. Neurospheres generally will grow better when plated at a higher density. Keep in mind the individual needs and growth rate of any particular cell line. Cell culture dishes with an Ultra-Low Attachment Surface could also be used to prevent cells for adhering and growing as a monolayer.

Weighing and assessment of the mice

Timing: 20 min

11. Before starting, it is important to weigh and check the mice. For our experiments, we use male or female athymic nude mice (age 5–7 weeks, average weight 20 g). Possible differences in weight should be accounted for between treatment groups. Also, a deviation from the expected weight may require adjustments in dosing of anesthesia, analgesics, luciferase reagent, and therapeutics.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
LV-Fluc-mCherry lentivirus vector	Maguire et al., 2008	n/a
Chemicals, peptides, and recombinant proteins		
(2-Hydroxypropyl)-β-cyclodextrin	Sigma-Aldrich	CAS # 128446-35-5
Articaine hydrochloride 4% and epinephrine 1:100,000	Septodont	CAS # 01A1400, NDC 3629004902
B-27 Supplement (50×), minus vitamin A	Gibco	CAS # 12587010
Buprenorphin hydrochloride 0.3 mg/mL	Hospira	NDC 00409-2012-32
CAY10566	Cayman and Glixx Lab	CAS # 944808-88-2
D-luciferin (150 mg/kg body weight)	Gold Biotechnology	CAS # 115144-35-9
DMEM (Dulbecco's modified Eagle's medium)	Corning	CAS # 10-013-CV
DMEM (Dulbecco's modified Eagle's medium)/Hams F-12 50/50 mix	Corning	CAS # 10-092-CV
DMSO	Sigma-Aldrich	CAS # 67-68-5
Dulbecco's phosphate-buffered saline (10×) without calcium, without magnesium (DPBS 10×)	Lonza	CAS # 17-515Q
Heparin sodium salt, for cell culture	Sigma-Aldrich	CAS # 9041-08-1
Isoflurane	Baxter	CAS # 1001936040
Mycoplasma elimination cocktail	abm	CAS # G398
Polybrene infection/transfection reagent	Sigma-Aldrich	CAS # TR-1003-G
Recombinant human EGF	abm	CAS # Z100135
Recombinant human FGF2 (bFGF)	abm	CAS # Z101456
StemPro Accutase cell dissociation reagent	Gibco	CAS # A11105-01
Trypan blue solution	Sigma-Aldrich	CAS # 72-57-1
Experimental models: cell lines		
Glioma cell line of interest	Primary glioblastoma stem-like cells (GSCs) used in the original study (Pinkham et al., 2019) were derived from a surgical specimen obtained from GBM patients under the appropriate Institutional Review Board approval	n/a
Experimental models: organisms/strains		
Male or female athymic nude mice, Hsd:Athymic Nude-Foxn1^{nu} (age 5-7 weeks, average weight 20 g)	Envigo	n/a

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Living Image software 4.3.1	PerkinElmer	https://www.perkinelmer.com/de/lab-products-and-services/resources/in-vivo-imaging-software-downloads.html#LivingImage
Other		
Bonewax wound closure - white bone wax, 2.5 g, 12/bx	Surgical Specialties	SKU # 2-104640-BX
COATED VICRYL (polyglactin 910) suture	Ethicon	CAS # VCP391H
Cotton tip wood shaft 6 inch sterile	McKesson	CAS # 24-106-2S
Digital Just for Mouse stereotaxic instrument	Stoelting	CAS # 51730D
Drill bit: 0.45mm for Neuroscience	Stoelting	CAS # 10-001-085
Epifluorescence microscope to detect mCherry/GFP	n/a	n/a
Hemocytometer or alternatively an automated cell counter	n/a	n/a
0.5 mL insulin syringe with 28G × 1/2 inch needle	BD	CAS # B-D329461Z
Microscope (bright-field) for cell counting	n/a	n/a
Mouse surgical kit	Kent Scientific	CAS # 13-005-204
Puralube veterinary ophthalmic ointment	Dechra Vet Products	CAS # 008897
Quintessential stereotaxic injector (QSI), dual	Stoelting	CAS # 53312
MultiPro Rotary Tool Model 395	Dremel	n/a
Sterile Disposable Scalpels	Stoelting	CAS # 10-000-853
Sterile prep pads 70% isopropyl alcohol antiseptic	Medline	CAS # MDS090735
Syringe 701N, volume 10 µL, needle size 26s ga (cone tip), needle L 51 mm (2 inch)	Hamilton	SKU # 28615-U
Xenogen IVIS 200 Imaging System	PerkinElmer	CAS # 124262

STEP-BY-STEP METHOD DETAILS

Preparation of the cells

Timing: 30 min

This step is critical for proper tumor seeding and tumor growth. A final quality check on the cells to be implanted should also be performed at this point before proceeding to surgery.

1. Collect all cells from one of the culture dishes you prepared the day before.
2. Spin down cells at 1,500 × *g* for 5 min and remove the supernatant.

Note: An accutase step might be required at this point in some cell lines (see preparation step 7b).

3. Resuspend cells in 1 mL of cell culture medium.
4. Pipette up and down to homogenize the cells into a single-cell suspension.
5. Count cells (see also preparation step 7e).
6. Spin down the appropriate volume of cell suspension containing the required number of cells at 1,500 × *g* for 5 min.

Note: The cell number is determined based on the number of mice to be implanted in the next 3 h (e.g., 10 mice) and the number of tumor cells to be implanted per mouse (e.g., 50,000

cells/mouse). We recommend preparing 1.5 times this amount per aliquot (see also preparation step 10).

7. Resuspend the cells in the appropriate volume of cold 1× phosphate-buffered saline (PBS) needed to achieve the desired concentration.
   a. We typically inject 2 μL of cell suspension in the brain of mice.

   Note: Injecting a high volume of cell suspension is not recommended as this would result in reflux and increase the chance of having an extracranial growth.

8. Transfer the cell suspension to a small tube.
9. Prepare additional resources.
   a. Prepare a small tube of ethanol (70%) to rinse the needle in between implantations.
   b. Prepare a small tube of 1× PBS to rinse the needle in between implantations.

   Note: The cells should not be kept on ice for an extended period of time (typically no longer than 3 h). It is recommended that you prepare fresh aliquots of cells every 3 h to ensure that the viability of the injected cells is not compromised. For each new aliquot, one of the plates prepared the day before can be used (this also facilitates counting as the concentration of the cell suspension should be similar).

Stereotactic implantation

Timing: 30 min per mouse, overlap possible

In this step, the actual implantation takes place. The implantation must take place under sterile conditions and must adhere to guidelines outlined in a protocol approved by Institutional Animal Care and Use Committee (IACUC).

10. Anesthesia and preparations
    a. Anesthetize mice with isoflurane.
    b. Turn on the heating pad.
    c. Once the mice are deeply anesthetized, place and fix the animal under the small animal stereotaxic frame. Note that this surgical set up has to be connected to an isoflurane chamber and the animal has to remain under anesthesia for the duration of the surgery.
    d. Administer analgesics.
11. Incision and localization of the injection site.
    a. Disinfect the skin.
    b. Incise the skin of the head with a scalpel over a length of 0.5–1 cm along the midline.
    c. Clean incision site with a sterile cotton tip.
    d. Identify the bregma.
    e. Point the needle tip to the bregma, then set x, y, and z coordinates to zero.
    f. Navigate to +0.5 anterior-posterior, +2.0 medio-lateral. Mark with a sterile pen.
12. Injection
    a. Take the needle out of the stereotactic frame.
    b. Fill the needle with 2 μL of cell suspension.
       i. First, rinse the needle with ethanol and PBS.
       ii. Make sure to mix the cell suspension before taking out the cells by gently tapping the tube with your finger.
       iii. Avoid having air bubbles in the syringe.
    c. Find the previously marked location on the skull and use a drill with a 0.45 mm burr drill bit (Stoelting) to pierce through the bone. Make sure not to exert too much pressure, in order to minimize the risk of causing brain injury once the drill head has penetrated the bone.

d. Reinsert the needle and navigate to −2.5 mm depth (dorso-ventral).
   i. Reinserting the syringe into the stereotactic frame might lead to a small drop of the cell suspension on the tip of the needle. Make sure to remove this to minimize the risk of tumor seeding and growth along the injection route.
e. Start the injection with 0.2 μL/min using a micropump installed into the stereotactic frame. Troubleshooting: clogging of the needle.
f. Removal of the needle
   i. Visually confirm that all the cell suspension volume has been injected.
   ii. After 60 s, start retracting the needle 0.3 mm every 30 s. This is important to prevent any reflux of the cells toward the skull.

13. Closing the wound
    a. Seal the bone with bone wax.
    b. Close the wound with surgical glue, stiches, or clamps.
14. Post procedure
    a. Administer analgesics.
    b. Place the mouse on a heat pad for recovery while closely monitoring for any signs of distress or difficulty breathing. Return the animal to its cage once it has recovered for anesthesia.

Imaging: monitoring of tumor growth by imaging Fluc bioluminescence activity

Timing: 20 min per cage, overlap possible

This step of the protocol enables the monitoring of tumor initiation and growth. The speed at which the cells engraft and grow in the mouse model varies considerably between different cell lines. Detectable tumors can develop within days or can take up to several weeks.

Tumor growth can be monitored by imaging Fluc bioluminescence activity using a cooled charge-coupled device (CCD) camera such as the Xenogen IVIS 200 Imaging System (PerkinElmer), after intraperitoneal injections of D-luciferin (150 mg/kg body weight). The Fluc signal, which is, in this case, a surrogate marker for tumor volume is quantitated using the Living Image software 4.3.1 (PerkinElmer).

15. Anesthetize mice with isoflurane.
16. Injection of D-luciferin
    a. Inject D-luciferin solution (reconstituted at 25 mg/mL in sterile PBS, administer 150 mg/kg body weight) intraperitoneally.
17. Wait 5–10 min to ensure proper biodistribution of the injected substrate into the animal. Acquire images (Figure 3).
18. Quantify the signal intensity with an appropriate software.

Troubleshooting: no signal

Note: For detailed information on bioluminescence imaging in mice, its limitations, and potential troubleshooting steps, see Badr (2014).

Note: The intensity of the bioluminescence signal can vary depending on the number of cells implanted as well as the transduction efficiency of the cells. Note that it is possible to perform cell sorting based on mCherry expression in order to achieve a pure population of luciferase-mCherry positive cells. We recommend starting the treatment once the bioluminescence signal from the brain tumor is at least 1.5-fold higher as compared to the background signal.

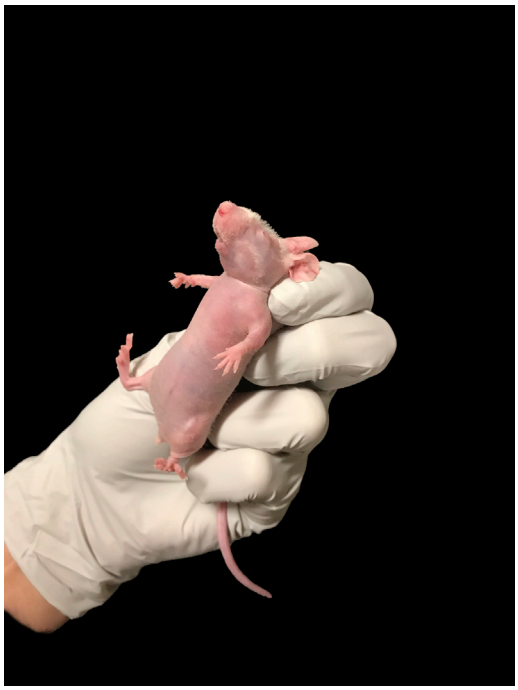


Figure 1. Proper handling of the mouse prior to IN injection

This picture shows how to hold the mouse in a half-sitting position with one hand.

Drug delivery: intranasal delivery of experimental compounds

Timing: 30 min

In this step, to overcome obstacles by various barriers of the CNS blood-brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barriers, the molecule of choice is delivered via nose-to-brain route by intranasal (IN) administration. The IN route of administration provides a non-invasive method for delivering therapeutics directly to the CNS. Several therapeutic compounds or biological agents such as peptides, proteins, oligonucleotides, nanoparticles, viral vectors, and stem cells can be delivered via the IN route (Dhuria et al., 2010; Oberoi et al., 2016; Bruinsmann et al., 2019).

This alternative drug delivery method has also other advantages over systemic administration such as an increased targeting to the brain and a reduced systemic toxicity.

19. Prepare the compounds for administration to the desired concentration. Make sure that the final volume does not exceed 10 μL for each nostril.
    a. Dissolving the drug in a 20% solution of (2-hydroxypropyl)- β-cyclodextrin can improve drug solubility, thus enhancing brain uptake after IN administration.
20. Drug administration
    a. Anesthetize mice with inhaled isoflurane.
    b. Confirm that the animal is deeply anesthetized by applying a firm toe pinch.
    c. Hold the anesthetized mouse with a skin grip while immobilizing the head and forelimbs (see Figure 1).
    d. Position the head-back in supine position with a 70°–90° tilt to maximize drug absorption from the nasal cavity and uptake into the brain, and to minimize drainage into the trachea and esophagus (see Figure 2).
    e. Using a pipette (P20), administer a small drop onto one nostril over a period of 30–60 s, thus allowing the drop to be snorted into the nasal cavity (see Figure 2). Check if normal breathing is restored before proceeding to the next step.

Figure 2. Administering the drug
This picture depicts the correct positioning of the pipette tip in order to administer the drug via the IN route. Make sure not to insert the pipette tip into the nostril or allow the pipette tip to touch the nostrils.

f. Repeat the previous step onto the contralateral nostril.
g. Repeat all the steps, alternating the nostrils until the 10 μL dose/nostril is completed.
h. Allow the mice to recover and regain consciousness over a heated pad before returning them back to their cages. The entire procedure takes ~30 min to be completed.

⚠ CRITICAL: Do not allow the pipette tip to touch the nostrils (Dhuria et al., 2010). Mouse are obligatory nose breathers (Kling, 2011; Grimaud and Murthy, 2018); inserting the pipette to administer the medication can cause unnecessary animal distress. Troubleshooting: Difficulty breathing or liquid leaking out of the nose immediately after injection

⚠ CRITICAL: Timing can be critical for the IN delivery. When a relatively high volume of liquid is injected too quickly, the result may be a difficulty in breathing (Dhuria et al., 2010) (which might cause the mice to wake up earlier; Troubleshooting: Mouse waking up during IN injection) or liquid leaking out of the nose immediately after injection. You should allow sufficient time for the mouse to sniff one drop at a time, before administering the next (Dhuria et al., 2010). On the other hand, if IN delivery is performed over an extended period of time, the anesthesia will wear off, and the mouse will wake up. Therefore, make sure the animal is deeply anesthetized prior to starting the IN administration of the drug. The aforementioned timeframe should serve as a reference point and can be adjusted according to individual experimental requirements. The maximum absorbance capacity of a mouse's nasal cavity is 0.032 cm^3 (Dhuria et al., 2010); working in a range of 10–30 μL volumes decreases the chances of lethal nostril occlusion.

For further reading on IN delivery techniques we refer to Dhuria et al., 2010; Lochhead and Thorne, 2012; Ullah et al., 2019.

Troubleshooting: Poor drug delivery to the brain

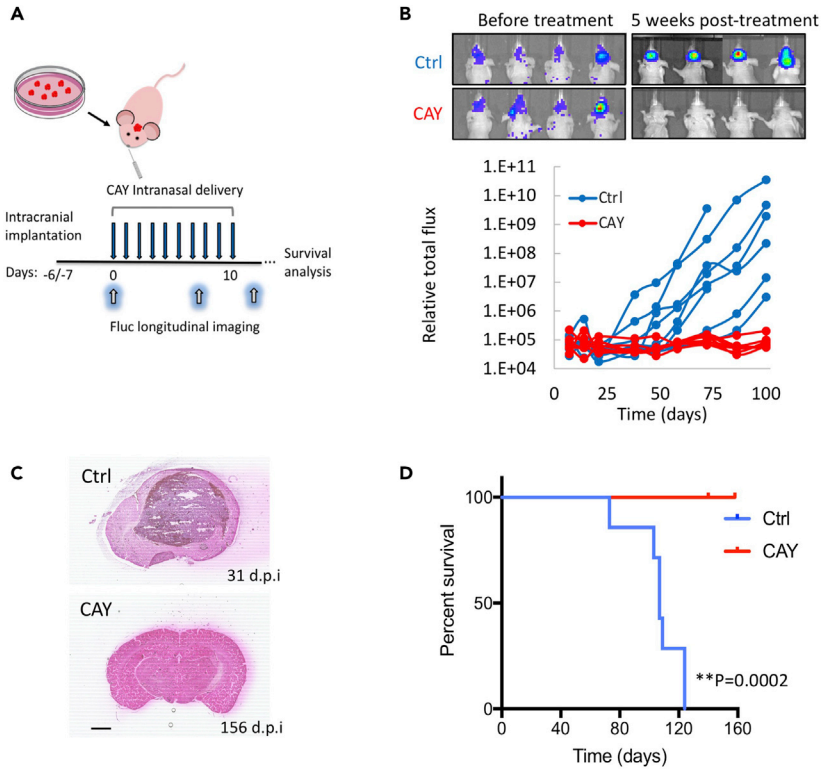


Figure 3. Therapeutic targeting of SCD1 in preclinical GBM mouse models

(A) Overview of experimental setup.

(B–D) Mice implanted with GSCs stably expressing Fluc (1×10^5; n = 8/group) were treated with vehicle or CAY (5 mg/kg). (B) Overtime Fluc imaging demonstrates the absence of tumor growth in all eight CAY-treated mice. (C) Brains from mice from the control group were isolated at day 25–31 post implantation (dpi). Similarly brains of the 2 remaining mice from the CAY-treated group were isolated at day 156 post implantation. Brain sections were analyzed by H&E staining. Micrographs from one representative mouse per group are shown. Scale bar, 1 mm. (D) Survival curves in both groups (p = 0.0002; two-sided log-rank test).

Adapted with permission from Pinkham et al. (2019).

EXPECTED OUTCOMES

Depending on the cell line used, tumors are usually established within days or weeks after implantation. It has been shown that drugs administered through the IN route can reach the brain within minutes (Ross et al., 2008), but the onset of measurable effects can vary depending on the administered compound, its pharmacological properties, and the dose applied. Based on our published results (Pinkham et al., 2019), the delivery of experimental GBM therapeutics via the IN route can be highly effective. Under our experimental conditions, we were able to demonstrate that a small molecule inhibitor of Stearoyl CoA Desaturase (CAY10566, 5 mg/kg) delivered through nasal instillation in GBM xenograft mouse models, effectively decreased tumor burden. This was assessed with Fluc imaging of brain tumors and Hematoxylin and eosin (H&E) staining of brain sections (Figure 3). Moreover, we also observed a significantly extended overall survival (Figure 3).

LIMITATIONS

Whenever a small number of tumor cells are implanted (e.g., when implanting a very aggressive and fast-growing GBM line) or when the injected tumor cells are poorly tumorigenic, a higher variation in tumor engraftment and/or tumor growth can be observed. This can be a confounding factor for treatment outcomes. Whenever possible, an attempt should be made to distribute the animals evenly among the groups based on the measured bioluminescence signal.

The implantation of the tumor cells should be standardized by injecting them through an automated pump in order to minimize inter-examiner variability. However, IN administration has a higher variability, since it cannot be standardized. It is advised that IN administration would be handled by the same examiner throughout the treatment process.

The delivery of experimental therapeutics to the brain via the IN route has several advantages as discussed earlier. While it could be very effective in determining whether an experimental compound can engage and target brain tumors in a mouse, it does not address potential systemic toxicity, nor does it provide information about the pharmacokinetic properties of a given compound, particularly its ability to cross the brain barriers. Further, it is unclear whether IN delivery provides a wide distribution of the injected compound throughout the brain. Therefore, this delivery method might not be optimal for all types of experimental glioma models (for example brain stem gliomas) and should be evaluated on case-by-case basis.

TROUBLESHOOTING

Problem 1

Clogging of the needle during intracranial injection (step 12e).

Potential solution

Rinse the needle several times with PBS and ethanol (95%–100%). If this does not help, remove the plunger and rinse the inside of the syringe by pushing ethanol into it with another syringe.

If the injection has already begun, we recommend excluding the mouse from the analysis, since it is no longer possible to reliably inject the exact number of cells, and accordingly, this would interfere with bioluminescence imaging quantification and survival outcome. To prevent clogging of the needle, rinse the needle after each injection, immediately after the injection has stopped and the needle has been retrieved. Clogging risk increases when injecting neurospheres that were not fully dissociated into a single-cell suspension.

Problem 2

No bioluminescence signal detected from tumors (step 18).

Potential solution

If no bioluminescence signal can be detected in the brain after imaging the mice, check whether the substrate (D-luciferin) is properly reconstituted and has been properly stored. If necessary, the injection should be repeated with a new aliquot.

In addition, whenever the appropriate equipment is available, it is possible to alternatively image fluorescence signal from the tumor (since cells express both Fluc and a fluorescent reporter). Of note, several in vivo imaging platforms such as Xenogen IVIS 200 Imaging System combine the ability to image bioluminescence and fluorescence.

If the signal is still undetected after the previous troubleshooting steps, imaging should be repeated on weekly basis to allow tumors to reach a detectable size.

Problem 3

Extracranial tumor growth (step 12).

Potential solution

Extracranial tumor growth can occur when cell suspension (fluid containing cells) is transferred to places outside the planned injection site in the brain. Wiping the needle with a sterile cotton tip before injection and wiping the injection site after injection can help remove any remaining cells outside the injection site, ensuring that no cells would seed outside the brain and form extracranial

tumors. Extracranial tumor growth can also be caused by injecting a very large volume of cell suspension, or by withdrawing the syringe too quickly after tumor cell injection. This may cause the cell suspension to be pushed back out of the implanted area. Extracranial tumor growth may become apparent at any time after implantation of the tumor cells. Practically, this may be noticed, for example, by an abnormally high bioluminescence signal or a visible lump on the head of the animal.

Problem 4

Poor drug delivery to the brain (step 20).

Potential solution

Poor drug delivery to the brain after IN administration can be caused by the protective barriers located in the nasal mucosa. To overcome issues related to poor drug delivery, some changes in drug formulation could be tested. For instance, drugs can be encapsulated into carriers other than cyclodextrin, such as microemulsions and nanoparticles to increase drug solubility and dispersion along the nose-to-brain route. Agents with mucoadhesive properties such as surface-engineered nanoparticles, efflux transporter inhibitors, and vasoconstrictors to reduce drug clearance by the mucociliary nasal cells, could increase the absorption time of the drug at the delivery site. This can potentially enhance the deliver into the CNS via olfactory and trigeminal nerves, systemic blood, or cerebrospinal fluid and lymphatic channels.

Head positioning is one of the major factors that influences the efficiency of drug delivery to the brain during IN administration. The correct grip handle with a dominant hand keeping the down- and forward head position, requires trained personnel to achieve the best dispersion and penetrance of the therapeutic for an efficient brain delivery and to avoid drug drainage into the airways, or to the esophagus.

Poor drug delivery to the brain may be evident by the fact that there is no measurable effect of the drug treatment which can be determined by bioluminescence imaging.

Problem 5

Difficulty breathing or liquid leaking out of the nose immediately after injection (step 20).

Potential solution

This is usually caused by either an excessive volume of liquid being injected into the nose, or by a rapid injection which does not allow enough time for inhalation and absorption of the injected liquid. Consider minimizing the injected volumes and/or injecting the solution at a slower rate. Also, make sure that the compound to be injected is fully soluble. Large particles or precipitates due to a poorly soluble compound can clog the nostrils or cause a major irritation.

Problem 6

Mouse waking up during IN injection (step 20).

Potential solution

To achieve a successful IN delivery, it is critical that the mouse is inert thus allowing the solution to be precisely administered into the nostril. Make sure the animal is deeply anesthetized prior to starting the IN administration of the drug. One reason for mice waking up during the procedure, can also be a difficulty in breathing. In this case, make sure one drop is sniffed in completely before administering the next.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Christian E. Badr, PhD (badr.christian@mgh.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No datasets or code were generated during this study.

ACKNOWLEDGMENTS

Research conducted in the Badr lab is supported by NIH/NCI K22CA197053, NIH/NINDS R01 NS113822, DoD Peer Reviewed Cancer Research CA191075, in part by Dana-Farber/Harvard Cancer Center 2P50 CA165962 SPORE in Brain Tumor Research, American Brain Tumor Association (ABTA) Discovery Grant supported by the Uncle Kory Foundation and Concern Foundation Conquer Cancer Now Award (to C.E.B.). K.M.E. was supported by a scholarship from the German Academic Exchange Service (DAAD). We acknowledge 1S10RR025504 Shared Instrumentation grant for the IVIS imaging system. We are very grateful to past and present members of the lab for their outstanding contribution to this work. We are grateful to Dr. Hiroaki Wakimoto (MGH) for providing primary GBM cells. The graphical abstract was created using BioRender.com.

AUTHOR CONTRIBUTIONS

Conceptualization, K.M.E., L.C., and C.E.B.; methodology, K.M.E., L.C., and C.E.B.; investigation, K.M.E., L.C., and C.E.B.; writing – original draft, K.M.E. and L.C.; writing – review & editing, K.M.E., L.C., and C.E.B.; visualization, K.M.E.; funding acquisition, C.E.B.; resources, C.E.B.; supervision, C.E.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Badr, C.E. (2014). Bioluminescent imaging. In Methods and Protocols, First Edition, C.E. Badr, ed. (Humana Press). Methods in Molecular Biology, 1098. https://doi.org/10.1007/978-1-62703-718-1.

Bruinsmann, F.A., Richter Vaz, G., de Cristo Soares Alves, A., Aguirre, T., Raffin Pohlmann, A., Stanisçuaski Guterres, S., and Sonvico, F. (2019). Nasal drug delivery of anticancer drugs for the treatment of glioblastoma: preclinical and clinical trials. Molecules *24*, 4312, https://doi.org/10.3390/molecules24234312.

Dhuria, S.V., Hanson, L.R., and Frey, W.H. (2010). Intranasal delivery to the central nervous system: mechanisms and experimental considerations. J. Pharm. Sci. *99*, 1654–1673, https://doi.org/10.1002/jps.21924.

Grimaud, J., and Murthy, V.N. (2018). How to monitor breathing in laboratory rodents: a review of the current methods. J. Neurophysiol. *120*, 624–632, https://doi.org/10.1152/jn.00708.2017.

Kling, M.A. (2011). A review of respiratory system anatomy, physiology, and disease in the mouse, rat, hamster, and gerbil. Vet. Clin. North Am. Exot. Anim. Pract. *14*, 287–337, https://doi.org/10.1016/j.cvex.2011.03.007.

Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N.M., Pastorino, S., Purow, B.W., Christopher, N., Zhang, W., et al. (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell *9*, 391–403, https://doi.org/10.1016/j.ccr.2006.03.030.

Lochhead, J.J., and Thorne, R.G. (2012). Intranasal delivery of biologics to the central nervous system. Adv. Drug Deliv. Rev. *64*, 614–628, https://doi.org/10.1016/j.addr.2011.11.002.

Maguire, C.A., Meijer, D.H., LeRoy, S.G., Tierney, L.A., Broekman, M.L., Costa, F.F., Breakefield, X.O., Stemmer-Rachamimov, A., and Sena-Esteves, M. (2008). Preventing growth of brain tumors by creating a zone of resistance. Mol. Ther. *16*, 1695–1702, https://doi.org/10.1038/mt.2008.168.

Oberoi, R.K., Parrish, K.E., Sio, T.T., Mittapalli, R.K., Elmquist, W.F., and Sarkaria, J.N. (2016). Strategies to improve delivery of anticancer drugs across the blood–brain barrier to treat glioblastoma. Neuro Oncol. *18*, 27–36, https://doi.org/10.1093/neuonc/nov164.

Pinkham, K., Park, D.J., Hashemiaghdam, A., Kirov, A.B., Adam, I., Rosiak, K., da Hora, C.C., Teng, J., Cheah, P.S., Carvalho, L., et al. (2019). Stearoyl CoA desaturase is essential for regulation of endoplasmic reticulum homeostasis and tumor growth in glioblastoma cancer stem cells. Stem Cell Reports *12*, 712–727, https://doi.org/10.1016/j.stemcr.2019.02.012.

Ross, T.M., Zuckermann, R.N., Reinhard, C., and Frey, W.H., 2nd (2008). Intranasal administration delivers peptoids to the rat central nervous system. Neurosci. Lett. *439*, 30–33, https://doi.org/10.1016/j.neulet.2008.04.097.

Ullah, I., Chung, K., Beloor, J., Lee, S.K., and Kumar, P. (2019). A positioning device for the placement of mice during intranasal siRNA delivery to the central nervous system. J. Vis. Exp. *150*, https://doi.org/10.3791/59201.