# Inhibition and eradication of human glioma with tumor-targeting *Salmonella typhimurium* in an orthotopic nude-mouse model

Masashi Momiyama,[1-3] Ming Zhao,[1] Hiroaki Kimura,[1] Benjamin Tran,[1] Takashi Chishima,[3] Michael Bouvet,[2] Itaru Endo[3] and Robert M. Hoffman[1,2,*]

[1]AntiCancer, Inc.; [2]Department of Surgery; University of California, San Diego; San Diego, CA USA; [3]Department of Gastroenterological Surgery; Yokohama City University; Yokohama, Kanagawa Japan

Malignant glioma tumors are the most common primary central nervous system tumors. Despite the multidisciplinary approach to treatment, prognosis remains poor. In this study, we demonstrated that the *Salmonella typhimurium* A1-R tumor-targeting strain can inhibit and eradicate human glioma in an orthotopic nude-mouse model. *S. typhimurium* A1-R was administered by injection through a craniotomy open-window or intravenously in nude mice. To establish the model, $2 \times 10^5$ U87-RFP human glioma cells were injected stereotactically into the mouse brain through the craniotomy open window. Two weeks after glioma-cell implantation, mice were treated with *S. typhimurium* A1-R [$2 \times 10^7$ CFU/200 μl intravenous injection (i.v.) or $1 \times 10^6$ CFU/1 μl intracranial injection (i.c.)] once a week for 3 weeks. Brain tumors were observed by fluorescence imaging through the craniotomy open window over time. *S. typhimurium* A1-R, administered i.c., inhibited brain tumor growth 7.6-fold compared with untreated mice (p = 0.009) and improved survival 73% (p = 0.001). Two of ten mice appeared to have their tumors eradicated. Intravenous administration of *S. typhimurium* A1-R was not effective. The craniotomy open window enabled observation of tumor growth in the brain in real time in both treated and untreated mice. The results of the present study demonstrate that bacterial therapy of brain cancer is a novel, effective and safe treatment strategy in a highly treatment-resistance cancer.

## Introduction

Malignant glioma tumors are the most common primary central nervous system (CNS) tumors and account for 78% of overall CNS tumors in adults.[1] The current standard of care for the treatment of malignant glioma are surgery, radiotherapy and chemotherapy.[2] Despite the multidisciplinary approach to treatment, prognosis remains poor and progression of disease is relatively common.[3-6]

There have been reports since the early 19th century that cancer patients who have been infected with bacteria have shown regression of their tumors.[7] At the end of the 19th century, Coley treated cancer patients with bacteria and later used extracts of the bacteria, called Coley's toxins, as a therapeutic. Coley's toxins showed significant efficacy in a large group of cancer patients.[8] Coley's toxins went out of favor after his death in the 1930s. Starting in the mid-20th century, there were preclinical studies on bacterial therapy of cancer and recently studies have been performed with obligate anaerobes such as Bifidobacterium and Clostridium in mouse models of cancer.[9-14] However, obligate anaerobes can only grow in the necrotic areas of tumors, which limits their efficacy. The facultative anaerobe *Salmonella typhimurium* (*S. typhimurium*) has also been attenuated for use as an anticancer

agent and tested in a clinical trial.[15] Although the Phase I clinical trial showed safety of this approach, tumor colonization by the bacteria was limited, perhaps due to over-attenuation.

We have previously developed *S. typhimurium* A1, which is auxotrophic (leu/arg-dependent) but receives sufficient nutritional support from tumor tissue. However, *S. typhimurium* A1-R does not continuously infect normal tissues.

To increase the tumor-targeting capability of *S. typhimurium* A1, the strain was re-isolated after infection into a human colon tumor growing in nude mice. The tumor-isolated strain, termed A1-R, demonstrated efficacy in treatment of mouse models of human breast cancer,[16] human prostate cancer,[17] fibrosarcoma,[18,19] pancreatic cancer,[20,21] spinal cord glioma[22] and lung cancer.[23,24]

In the present study, we demonstrate that *S. typhimurium* A1-R, administered by intracranial injection through a craniotomy open window, can effectively treat human glioma in an orthotopic nude-mouse model.

## Results and Discussion

**Real-time in vivo imaging of U87-red fluorescent protein (RFP) glioma growing orthotopically in nude mice.** RFP-expressing
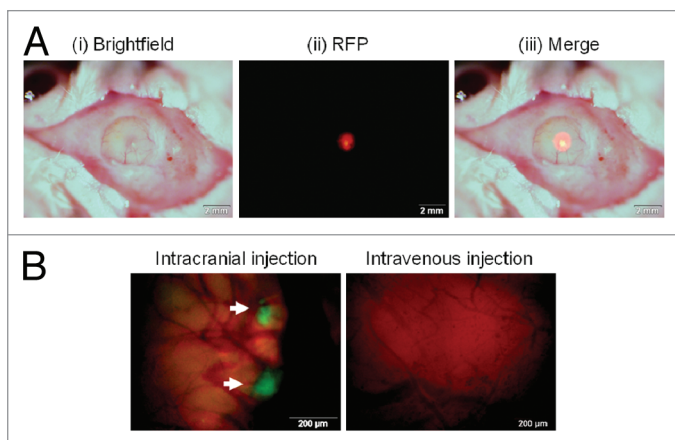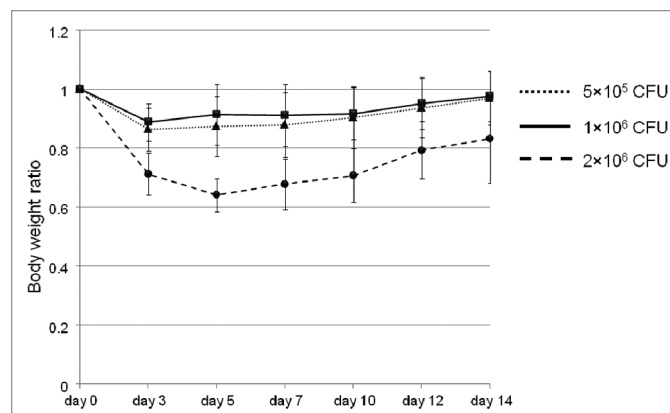
Figure 1. RFP-expressing U87 glioma tumor and GFP-labeled *S. typhimurium* A1-R in the brain observed through the craniotomy open window in live mice. (A) (i) Using a skin biopsy punch, a 4 mm diameter craniotomy was made over the right parietal bone. (ii) RFP-expressing U87 tumor growing in the brain was observed with florescence imaging through the craniotomy open window. (iii) Merged image of bright field and florescence image. (B) Colonies of GFP-labeled *S. typhimurium* A1-R (arrows) were observed in the U87-RFP brain tumor treated by intracranial injection of *S. typhimurium* A1-R (left column). No *S. typhimurium* colonies were found in the tumor treated with intravenous injection (right column). Scale bars: (A) 2 mm; (B) 200 μm.



Figure 2. Dose-limiting toxicity determination of *S. typhimurium* A1-R in the brain. Mice without tumors were tested for determination of *S. typhimurium* A1-R dose-limiting toxicity using intracranial injection. The mice were injected intracranially weekly with different doses of the bacteria (5 x 10⁵ CFU/μl, 1 x 10⁶ CFU/μl and 2 x 10⁶ CFU/μl). The body weight ratio was compared within three groups to decide the optional dosage of the bacteria. In the 5 x 10⁵ CFU/μl and 1 x 10⁶ CFU/μl groups, the maximum weight loss was 13.7% and 11.2%, respectively, and weight recovered by day 14 in both groups. In the 2 x 10⁶ CFU/μl group, however, the maximum weight loss was 35.9 on day 5, and on day 14, the weight loss was still 12.9%. The optimal dose of *S. typhimurium* A1-R in the brain was therefore determined to be 1 x 10⁶ CFU/μl.

U87 glioma tumors in the brain were observed through the craniotomy open window (**Fig. 1A**). The craniotomy open window enables imaging tumor growth in the brain over time while keeping animals alive and without further invasive procedures. Colonies of GFP-labeled *S. typhimurium* A1-R were observed in the U87-RFP brain tumor after cranial injection, but no bacterial colonies were found in the tumor treated with intravenous injection of *S. typhimurium* A1-R (**Fig. 1B**).

**Dose-limiting toxicity of *S. typhimurium* A1-R administered by intracranial injection.** Mice without tumors were tested for determination of *S. typhimurium* A1-R dose-limiting toxicity after intracranial administration of the bacteria. The mice were injected intracranially weekly with different doses of the bacteria (5 x 10⁵ CFU/μl, 1 x 10⁶ CFU/μl and 2 x 10⁶ CFU/μl). The body weight ratio (body weight at each day/body weight at day 0) was compared in the three groups to determine toxicity (**Fig. 2**). In the 5 x 10⁵ CFU/μl and 1 x 10⁶ CFU/μl groups, the maximum weight loss was 13.7% and 11.2%, respectively, and weight recovered by day 14 in both groups. In the 2 x 10⁶ CFU/μl group, however, the maximum weight loss was 35.9% on day 5, exceeding 20% weight loss, and by day 14, body weight did not recover. The treatment dose of *S. typhimurium* A1-R for brain cancer was therefore chosen as 1 x 10⁶ CFU/μl.

**Evaluation of *S. typhimurium* A1-R therapeutic brain cancer efficacy over time.** Mice in the treatment group were given *S. typhimurium* A1-R weekly by intravenous injection (i.v.) or intracranial injection (i.c.) for 3 weeks, beginning 14 d after tumor transplantation. Mice were administered the same volume of PBS as the untreated group (control). Tumor size was measured using RFP imaging at days 14, 21, 28 and 35 (**Fig. 3A**).

The bacteria therapy was evaluated by tumor volume, which was measured using fluorescence imaging. Efficacy of the bacteria treatment is shown in **Figure 3B**. Average tumor size on day 14 (the day *S. typhimurium* A1-R was administered) was 1.81 mm³ in the control group, 0.74 mm³ in the intravenous group, and 0.68 mm³ in the intracranial group. There was no significant difference among three groups. Tumor volume in the intracranial-treatment group was 2.87 mm³ and 11.5 mm³ on days 28 and 35, respectively. These volumes were significantly smaller than in the other two groups (on day 28, vs. control: 29.0 mm³, p = 0.034; vs. intravenous: 8.22 mm³, p = 0.048; on day 35, vs. control: 87.6 mm³, p = 0.009; vs. intravenous: 58.1 mm³, p = 0.014). However, there was no significant difference in tumor volume between the control group and the intravenous group (at day 21, p = 0.233; at day 28, p = 0.075; and at day 35, p = 0.265).

**Survival efficacy of *S. typhimurium* A1-R therapy on U87-RFP glioma.** Survival efficacy of *S. typhimurium* A1-R therapy in the orthotopic model is shown in **Figure 4**. Death from disease was rapid in the control group and intravenous group. Their median survival times were 37 and 43 d, respectively. Treatment with intracranial injection significantly prolonged median survival to 64 d (p = 0.001). Two out of seven mice treated by intracranial injection survived at day 80.

Intracranial injection of *S. typhimurium* was significantly more effective and resulted in prolonged survival in mice compared with intravenous injection (**Figs. 3 and 4**). It appears that *S. typhimurium* poorly penetrates the blood-brain barrier. Unlike systemic therapy, intracranial injection of *S. typhimurium* A1-R can bypass the blood-brain barrier, which is the major obstacle in treatment of brain cancer. Intracranial administration delivers *S. typhimurium* A1-R directly to the brain tumor.
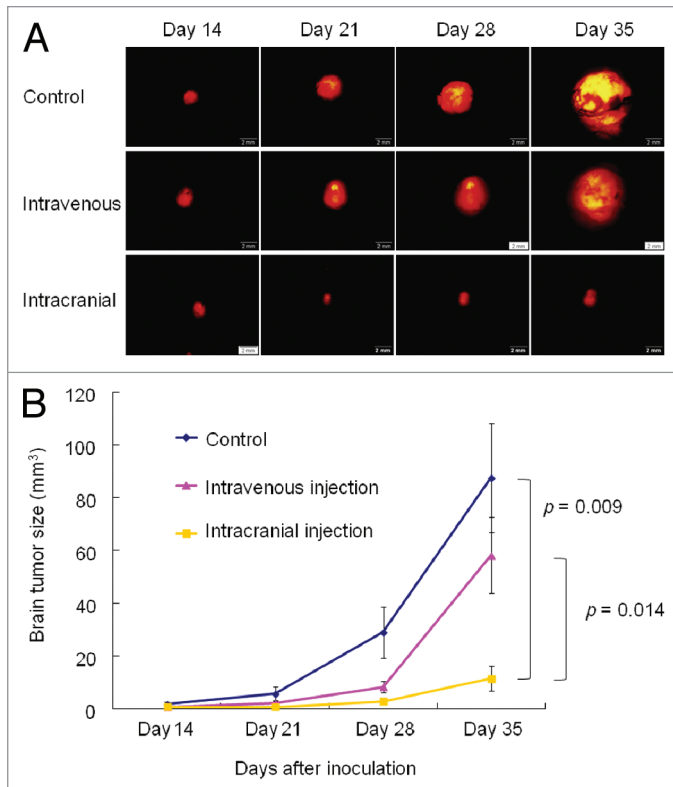
**Figure 3.** Real-time, in vivo imaging of U87-RFP glioma growing orthotopically in nude mice and evaluation of *S. typhimurium* A1-R therapeutic efficacy over time. (A) Representative images of tumor growth in untreated group (control); treated with intravenous injection (i.v. injection); and treated by cranial injection of *S. typhimurium* A1-R (intracranial injection). Mice in the treatment groups were given *S. typhimurium* A1-R weekly by i.v. injection or intracranial injection for 3 weeks, beginning 14 d after tumor transplantation. Tumor size was measured using RFP imaging at days 14, 21, 28 and 35. Scale bars: 2 mm. (B) Tumor volumes in each group were compared. Tumor volume in the intracranial-injection group was significantly smaller than in the other two groups (at day 35, vs. control: p = 0.009; vs. intravenous: p = 0.014). However, there was no significant difference in tumor volume between the control group and intravenous-injection group. Seven mice were used in each group. The experimental data are expressed as the mean ± SD. Statistical analysis was performed using the Student t-test.

*S. typhimurium* A1-R is auxotrophic for both leucine and arginine, which appears to preclude these bacteria from mounting a sustained infection in normal tissue. Tumors, however, appear to be able to supply these amino acids sufficiently and enable *S. typhimurium* A1-R to grow in and kill tumors.[25] *S. typhimurium* A1-R was selected for increased virulence by passage through a growing tumor in a mouse.[16]

Weekly administration of bacteria at the appropriate dose by intracranial delivery had no apparent adverse effects (such as meningitis, encephalitis). The mice tolerated the treatment well and appeared healthy during the experiment. These safety results suggest that direct cranial injection of bacteria in patients with brain cancer could be a clinically effective method for treatment of this highly drug-resistance disease.

In conclusion, we have demonstrated that *S. typhimurium* A1-R monotherapy rapidly inhibits human U87 glioma in the
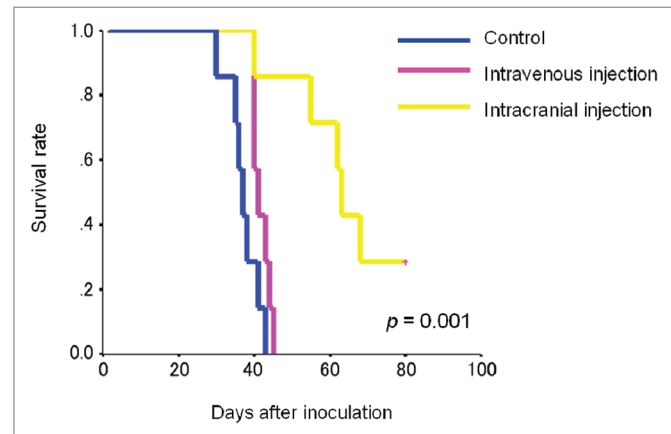


**Figure 4.** Survival efficacy of *S. typhimurium* A1-R therapy on U87-RFP glioma growing orthotopically in nude mice. Treatment by intracranial injection significantly prolonged survival. Median survival increased from 37 and 43 d in the control group and intravenous injection group, respectively, to 64 d in the intracranial injection group (p = 0.001). Two out of seven mice treated intracranial injection with the bacteria survived at day 80. Seven mice were used in each group. Statistical analysis was performed using the Kaplan-Meier analysis with log-rank test.

brain of mice in an orthotopic brain tumor model. U87 cancer cells were highly sensitive to *S. typhimurium* A1-R therapy, which prolonged survival of the mice. The craniotomy open window also enables imaging of tumor growth in the brain over time, keeping animals alive, and enables evaluation of the efficacy of a treatment in real time. Bacterial therapy of brain cancer is a novel and effective treatment strategy for this disease.

Our results also suggest potential use of *S. typhimurium* A1-R to target[26] glioma properties such as delivery of glioma-suppressing microRNAs;[27] targeting enzymes involved with aerobic glycolysis;[28] targeting the hypoxic glioma tumor microenvironment[29] and targeting the Tie2/TEK tyrosine kinase receptor.[30]

## Materials and Methods

**Establishment of RFP labeled cancer cell line.** For RFP gene transduction of cancer cells, 70% confluent human glioma (U87) cells were used. In brief, cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI 1640 (Irvine Scientific) containing 10% fetal bovine serum (FBS) (Hyclone Laboratories) for 72 h. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 h post-transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 μg/ml of G418. The level of G418 was increased stepwise up to 800 μg/ml.

**Cell culture.** U87 cells were maintained in DMEM medium (Hyclone Laboratories) supplemented with 10% FBS. The cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. The cells were collected after trypsinization and stained with trypan blue (Sigma-Aldrich). Only viable cells were counted with a hemocytometer (Hausser Scientific).

**Green fluorescent protein (GFP) gene transfection of *S. typhimurium*.** *S. typhimurium* (ATCC 14028) was grown at

37°C to mid-logarithmic phase in liquid Luria Bertani (LB) and harvested at 4°C. Bacteria ($2.0 \times 10^8$), in 40 μl 10% glycerol, were mixed with 2 μl pEGFP vector (Clontech). The preparation was placed on ice for 5 min before electroporation, using the Gene Pulser apparatus (Bio-Rad) according to the manufacturer's instructions. Electroporation was performed at 1.8 kV, with the pulse controller at 1,000 Ω parallel resistance.[31] Bacteria engineered to express light-emitting proteins have been shown to be readily visualized in tumors.[32,33]

**Induction of bacterial mutations with nitrosoguanidine and selection for auxotrophs of *S. typhimurium*-GFP.** Freshly prepared nitrosoguanidine (NTG; 1 mg/ml in sterile water) was added to the washed culture to a final concentration of 100 μg/ml in Tris-maleic acid buffer (pH 6.0). *S. typhimurium*-GFP were incubated with NTG for 30 min. NTG-treated cells were grown in nutrient broth to express any mutations that had been induced. Bacterial colonies were replica-plated in supplemented minimal agar plates, containing specific amino acids in order to identify auxotrophic requirements. Auxotroph A1, which required leucine and arginine, was initially isolated.[25]

**Selection of the high-tumor-targeting variant *S. typhimurium* A1-R.** Selection of a high-tumor-targeting variant of *S. typhimurium* A1-GFP was performed as follows: A1 bacteria were injected into the tail vein of an HT-29 human colon tumor-bearing nude mouse. Three days after infection, tumor tissue was removed from the infected animal. Tumor tissue was then homogenized and diluted with phosphate-buffered saline (PBS). The resulting supernatant of the tumor tissue was cultured in LB agar plates at 37°C overnight. The bacterial colony with the brightest green fluorescence was selected and cultured in 5 ml of LB medium. This strain was termed A1-R.[16]

**Growth of *S. typhimurium* A1-R for treatment.** *S. typhimurium* A1-R were grown overnight in LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS and then diluted in PBS. Bacteria were then ready for injection in mice.[16]

**Animals.** Athymic NCR nude mice (*nu/nu*) at 4–6 weeks of age were used in this study. The breeding pairs were obtained from Charles River Laboratories. Mice were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principals and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under PHS Assurance Number A3873-1.

**Craniotomy open window.** Mice were anesthetized with a ketamine mixture (10 μl ketamine HCL, 7.6 μl xylazine, 2.4 μl acepromazine maleate and 10 μl $H_2O$) via s.c. injection. After fixing the mice in a prone position, a 1.5 cm incision was made directly down the midline of the scalp. The scalp was retracted and the skull was exposed. Using a skin biopsy punch (Acuderm Inc.), a 4 mm diameter craniotomy was made over the right parietal bone (**Fig. 1A**). The bone fragment was removed carefully in order not to injure the meninges and brain tissue. The craniotomy open window was covered only by the scalp. Thus, only scalp retraction was needed in order to image tumor growth or inject *S. typhimurium* in the brain. The incision was then closed with 6–0 surgical suture (ETHICON, Inc.). All mice were kept in an oxygenated warmed chamber until they recovered from anesthesia.

**Stereotactic injection.** The mice were anesthetized with a ketamine mixture via s.c. injection. After the craniotomy open window was made, 1 μl of a suspension containing $2 \times 10^5$ U87-RFP cells was injected stereotactically into the mouse brain using a 10 μl Hamilton syringe. Cells were injected in the middle of the craniotomy open window to a depth of 1 mm.

**Bacterial therapy in the brain tumor model.** Two weeks after inoculation, mice were treated with *S. typhimurium* A1-R ($2 \times 10^7$ CFU/200 μl PBS intravenous injection from the tail vein or $1 \times 10^6$ CFU/1 μl PBS intracranial injection through the craniotomy open window) once a week for 3 weeks. Mice were administered the same volume of PBS as the untreated control group. After administration of *S. typhimurium* A1-R, fluorescence imaging (please see below) was performed (**Fig. 1A**) and changes in the diameters of the RFP-expressing tumors were recorded each week for 3 weeks. Tumor diameters were measured each week after *S. typhimurium* A1-R administration. Tumor volume was calculated by the formula (width$^2$ x length x 0.5). Seven mice were used in each group.

**Fluorescence imaging.** The Olympus OV100 Small Animal Imaging System (Olympus), containing an MT-20 light source (Olympus Biosystems) and DP70 CCD camera (Olympus), was used for imaging live mice.[34] High-resolution images were captured directly on a personal computer (Fujitsu Siemens Computers). Images were analyzed with the use of Cell$^R$ software (Olympus Biosystems).

**Survival analysis.** The experimental data are expressed as the mean ± SD. Statistical analysis was performed using the Student t-test. Kaplan-Meier analysis with a log-rank test was used to determine survival and difference between treatment groups. A p-value less than 0.05 was used to indicate a significant difference.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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