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## Priority Report

**Effects of Photoacoustic Imaging and Photothermal Ablation Therapy Mediated by Targeted Hollow Gold Nanospheres in an Orthotopic Mouse Xenograft Model of Glioma**Wei Lu<sup>1</sup>, Marites P. Melancon<sup>1,2</sup>, Chiyi Xiong<sup>1</sup>, Qian Huang<sup>1</sup>, Andrew Elliott<sup>2</sup>, Shaoli Song<sup>1</sup>, Rui Zhang<sup>1</sup>, Leo G. Flores II<sup>1</sup>, Juri G. Gelovani<sup>1</sup>, Lihong V. Wang<sup>3</sup>, Geng Ku<sup>1</sup>, R. Jason Stafford<sup>2</sup>, and Chun Li<sup>1</sup>**Abstract**

Advancements in nanotechnology have made it possible to create multifunctional nanostructures that can be used simultaneously to image and treat cancers. For example, hollow gold nanospheres (HAuNS) have been shown to generate intense photoacoustic signals and induce efficient photothermal ablation (PTA) therapy. In this study, we used photoacoustic tomography, a hybrid imaging modality, to assess the intravenous delivery of HAuNS targeted to integrins that are overexpressed in both glioma and angiogenic blood vessels in a mouse model of glioma. Mice were then treated with near-infrared laser, which elevated tumor temperature by 20.7°C. We found that PTA treatment significantly prolonged the survival of tumor-bearing mice. Taken together, these results show the feasibility of using a single nanostructure for image-guided local tumor PTA therapy with photoacoustic molecular imaging. *Cancer Res*; 71(19): 6116–21. ©2011 AACR.

**Introduction**

With nanotechnology, it is possible to create multifunctional nanostructures capable of mediating diagnostic imaging, treatment, and monitoring of therapeutic response. For example, pegylated gold nanorods were used for both X-ray computed tomography and photothermal ablation (PTA; ref. 1). This approach, sometimes referred to as "theranostics," holds great promise for cancer diagnosis and therapy. Photoacoustic tomography (PAT) is a hybrid imaging modality that detects absorbed photons ultrasonically through the photoacoustic effect (2). PAT is capable of detecting optical contrast agents with high sensitivity and specificity (3–5). With the metal nanocomposites, photoacoustic imaging and temperature measurement for photothermal cancer therapy was investigated in tissue-mimicking phantoms and excised animal tissues (6).

We hypothesize that PAT is an accurate and sensitive imaging modality for cancer diagnosis and for assessing targeted delivery to brain tumors of gold nanostructures used for PTA therapy. Silica-cored gold nanoshells, which are under clinical investigation, have been shown to mediate effective PTA therapy and improve survival in a subcutaneous glioma model in mice (7). In this work, we showed that intravenous injection of hollow gold nanospheres (HAuNS) targeted to integrins that are overexpressed in both glioma and angiogenic blood vessels (4, 8, 9), permitted PAT of orthotopically inoculated U87 glioma in nude mice, and mediated selective antitumor effect when mice were irradiated with near-infrared (NIR) laser. Although there have been numerous reports on theranostic applications of cancer nanotechnology, to the best of our knowledge, this is the first report to show simultaneous molecular PAT and PTA therapy for cancer with a single platform of targeted nanoparticles.

**Materials and Methods****Nanoparticle synthesis**

HAuNS were synthesized as previously described (10). Cyclic RGD peptide c(KRGDf) was synthesized manually by PL-DMA resin and *N*<sup>ε</sup>-Fmoc chemistry (11). Cyclic RGD peptides have been used for imaging and treatment of glioma (4, 8, 9) because of their high binding affinity to integrin receptors such as  $\alpha_v\beta_3$  receptors ( $IC_{50} = 2\text{--}40$  nmol/L; refs. 12, 13). The peptide was conjugated to one terminus of a heterodifunctional polyethylene glycol (PEG) precursor, *N*-hydroxysuccinimidyl-PEG-S-acetylthioacetate (NHS-PEG-SATA; molecular weight 5,000), through its  $\epsilon$ -amine of lysine residue and activated ester in NHS-PEG-SATA. The sulfhydryl group on the other terminus of c(KRGDf)-PEG-SATA was released by treatment with

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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0.5 mol/L hydroxylamine in PBS (Supplementary Fig. S1). HAuNS ( $8.5 \times 10^{12}$  particles/mL) were then added to argon-purged aqueous solution containing c(KRGDf)-PEG-SH (50  $\mu\text{g/mL}$ ) and PEG-SH (500  $\mu\text{g/mL}$ ; Sigma) and the solution was stirred overnight at room temperature to give c(KRGDf)-PEG-HAuNS (Fig. 1A). PEG-SH was conjugated to HAuNS similarly to give PEG-HAuNS (nonspecific control).

### Photoacoustic imaging

PAT of mouse brain inoculated with human glioblastoma stably transfected with *luciferase* gene (U87-TGL) was carried out as we previously described (3). A brief description of the PAT technique is provided in the Supplementary Data. The cell line provided by Dr. Juri G. Gelovani (University of Texas) in January 2008 was validated by short tandem repeat (STR) DNA fingerprinting by the Characterized Cell Line Core Facility at The University of Texas MD Anderson Cancer Center on April 12, 2011. The STR profiles were compared with known ATCC fingerprints, and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (<http://bioinformatics.istge.it/clima/>). The STR profiles matched known DNA fingerprints of U87-MG.

On day 8 after tumor inoculation, mice were imaged by a prototype PAT scanner. Mice were then injected i.v. with c(KRGDf)-PEG-HAuNS or PEG-HAuNS ( $2.5 \times 10^{11}$  particles per mouse). Contrast-enhanced photoacoustic images were acquired 24 hours after nanoparticle injection. Imaging data were reconstructed by using modified delay-and-sum back-projection reconstruction algorithm. After data acquisition, mice were sacrificed by overexposure to  $\text{CO}_2$ . Open-skull

surgery was conducted to permit photography of the brain tumors.

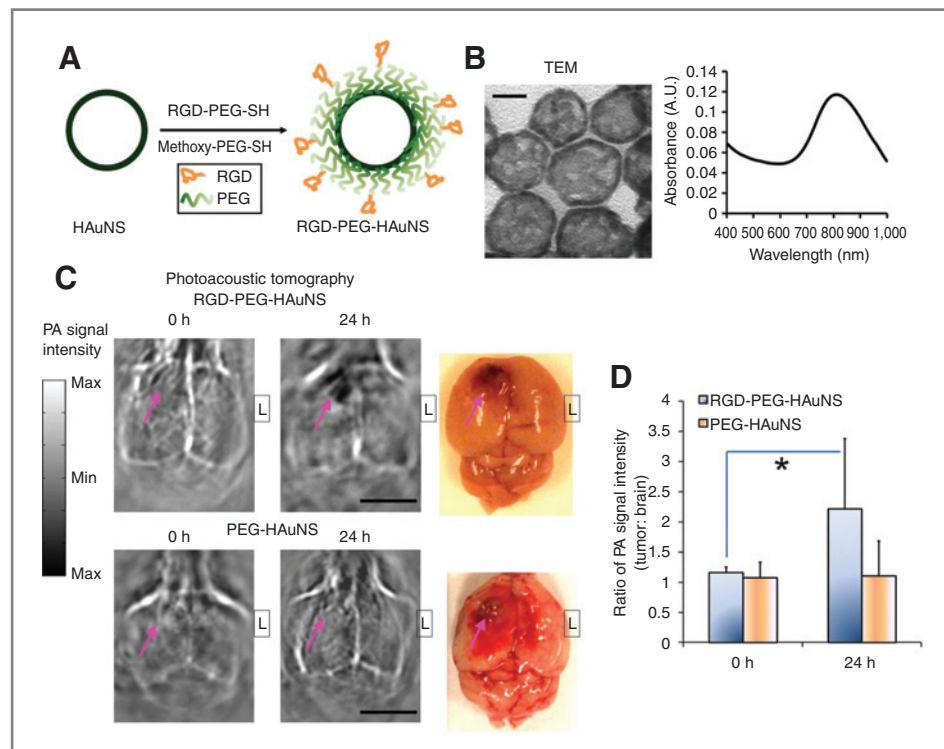
### Micro-positron emission tomography

To further confirm the accuracy of PAT imaging of U87 tumors, c(KRGDf)-PEG-HAuNS were labeled with the positron emitter  $^{64}\text{Cu}$  ( $t_{1/2} = 12.7$  hours; ref. 14). Tumor-bearing mice were injected i.v. with  $^{64}\text{Cu}$ -labeled c(KRGDf)-PEG-HAuNS or  $^{64}\text{Cu}$ -labeled PEG-HAuNS at a dose of 7.5 mCi/kg ( $n = 3$  per agent). Twenty-four hours later, mice were anesthetized with 2% isoflurane (Baxter). Mice were subjected to a 7-minute micro-computed tomography ( $\mu\text{CT}$ ) scan followed by a 20-minute micro-positron emission tomography ( $\mu\text{PET}$ ) scan with an Inveon  $\mu\text{PET/CT}$  Scanner (Siemens). The  $\mu\text{PET/CT}$  images were generated separately and then fused by Inveon Research Workplace version 3.0 (Siemens).

### Photothermal therapy

On day 8 after tumor inoculation, mice were injected i.v. with D-luciferin (4 mg/kg) for bioluminescence imaging. Mice were randomly allocated into 5 groups of 15 mice each. Groups I and IV were injected i.v. with c(KRGDf)-PEG-HAuNS, group II with PEG-HAuNS (both  $2.5 \times 10^{11}$  particles per mouse), and groups III and V with saline. Groups I to III were also treated with NIR laser irradiation (16  $\text{W/cm}^2$ , 3 minutes, 808 nm) 24 hours after nanoparticle injection. In each group, 5 mice were subjected to luciferase bioluminescence imaging at various times after nanoparticle injection to measure tumor burden (response to PTA therapy), and 10 mice were subjected to

Figure 1. A, scheme for c(KRGDf)-PEG-HAuNS bioconjugation. B, c(KRGDf)-PEG-HAuNS characteristics on transmission electron microscopy (bar, 20 nm) and UV-visible spectrum (measured in water). C, PAT images of U87 human glioma in mouse brains before (0 hour) and 24 hours after i.v. injection of nanoparticles (bar, 5 mm). Photographs of corresponding mouse brains were used to confirm tumor location. Arrows, locations of tumors; L, left. D, photoacoustic (PA) signal intensity ratio of tumor-to-contralateral brain in mice before (0 hour) and 24 hours after injection of HAuNS. Data are presented as mean  $\pm$  SD. c(KRGDf)-PEG-HAuNS group,  $n = 5$ ; PEG-HAuNS group,  $n = 4$ . \*, significant difference between precontrast and 24-hour postcontrast groups ( $P < 0.05$ ).



survival monitoring. Survival data were presented by Kaplan–Meier plots and analyzed by a log-rank test.  $P < 0.05$  was considered statistically significant.

## Results

### PAT permits visualization of U87 gliomas in intact nude mice

Transmission electron microscopy showed that c(KRGDf)-PEG-HAuNS were spherical, had hollow interiors, had average diameter of approximately 40 nm, and had an absorption peak tuned to approximately 800 nm (Fig. 1B). We previously showed that the molar extinction coefficient associated with the optical absorption cross-section of HAuNS [ $1.4 \times 10^{11}$  (mol/L) $^{-1}$ .cm $^{-1}$ ] is much higher than that of hemoglobin [ $\sim 1 \times 10^3$  (mol/L) $^{-1}$ .cm $^{-1}$ ]. Indeed, PEG-HAuNS at a concentration as low as 20 pmol/L displayed the same optical absorption as hemoglobin at its typical blood concentration of 2.3 mmol/L (3). The strong absorption of PEG-HAuNS in the NIR region permitted intense contrast in PAT.

Images of mice with U87 glioma before and after i.v. injection of c(KRGDf)-PEG-HAuNS or PEG-HAuNS are shown in Fig. 1C. Precontrast PAT only showed the normal brain vasculature. There was a low signal-to-background ratio between tumor and normal brain with no significant difference in photoacoustic signal intensities (Fig. 1D). However, 24 hours after injection of c(KRGDf)-PEG-HAuNS, PAT clearly revealed brain tumor, and the tumor location on PAT correlated with mouse brain anatomy. Quantitative analysis confirmed that the mean contrast-enhanced photoacoustic signal ratio of tumor-to-contralateral normal brain 24 hours after c(KRGDf)-PEG-HAuNS injection was approximately twice as high as that obtained from precontrast images ( $P = 0.0375$ ). In contrast, 24 hours after injection of PEG-HAuNS, there was no change in tumor-to-brain photoacoustic ratio compared with that obtained from precontrast PAT images ( $P = 0.4677$ ). These data supported that selective

accumulation of c(KRGDf)-PEG-HAuNS enhanced PAT of U87 glioma.

### $\mu$ PET/CT confirms accuracy of PAT imaging of U87 gliomas with c(KRGDf)-PEG-HAuNS

Twenty-four hours after i.v. injection,  $^{64}\text{Cu}$ -labeled c(KRGDf)-PEG-HAuNS showed 2.9-fold higher uptake than  $^{64}\text{Cu}$ -labeled PEG-HAuNS in U87 tumors [ $1.12 \pm 0.22$  percentage of injected dose per gram of tissue (%ID/g) vs.  $0.38 \pm 0.12\%$  ID/g; Fig. 2; Supplementary Fig. S2], and autoradiography showed higher radioactivity in tumor with c(KRGDf)-PEG-HAuNS than PEG-HAuNS. In addition to integrin  $\alpha_v\beta_3$ , recent study showed that c(KRGDf) may also interact with other integrins such as  $\alpha_v\beta_5$  (15). Here, we used immunohistochemical staining of integrin  $\alpha_v\beta_3$  receptors in tumors to confirm HAuNS localization. The intracranial distribution of radioactivity matched well with the site of tumor inoculation as well as the distribution of integrin  $\alpha_v\beta_3$ . Selective uptake of c(KRGDf)-PEG-HAuNS in U87 tumors was further supported by microscopy, which revealed greater accumulation of c(KRGDf)-PEG-HAuNS than PEG-HAuNS in tumors (Fig. 2).

### Injection of c(KRGDf)-PEG-HAuNS plus NIR laser irradiation elevates temperature of U87 gliomas

Representative magnetic resonance temperature imaging (MRTI) maps of mouse brains at the end of laser irradiation are shown in Fig. 3A. Dynamic temperature curves based on MRTI analysis revealed that NIR laser irradiation in mice injected with c(KRGDf)-PEG-HAuNS reached a maximum temperature of  $57.75 \pm 0.46^\circ\text{C}$  (Fig. 3B), which is more than the threshold of temperature of  $54^\circ\text{C}$  needed for irreversible cell damage (16, 17). In contrast, NIR laser irradiation in mice injected with nontargeted PEG-HAuNS or saline resulted in maximum temperatures of  $48.14 \pm 0.12^\circ\text{C}$  and  $41.65 \pm 0.09^\circ\text{C}$ , respectively, which were insufficient to induce irreversible cell damage (Fig. 3B).

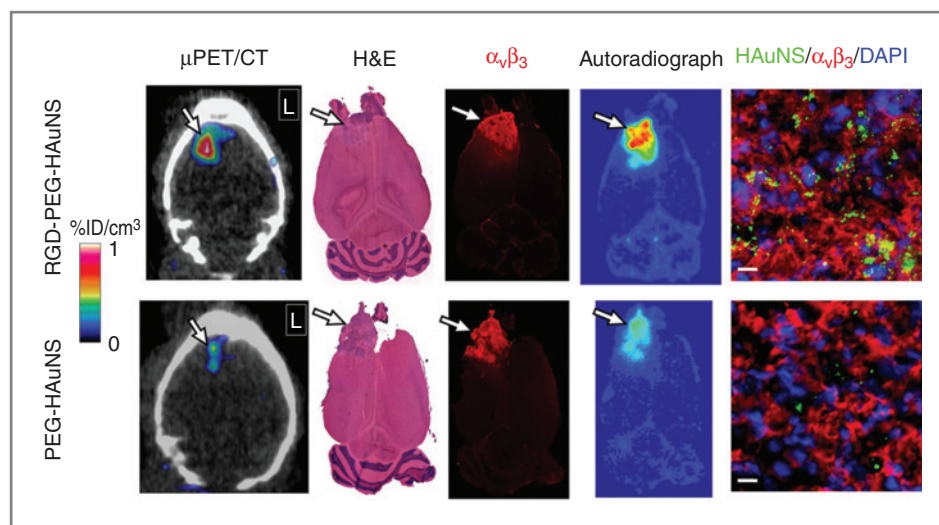
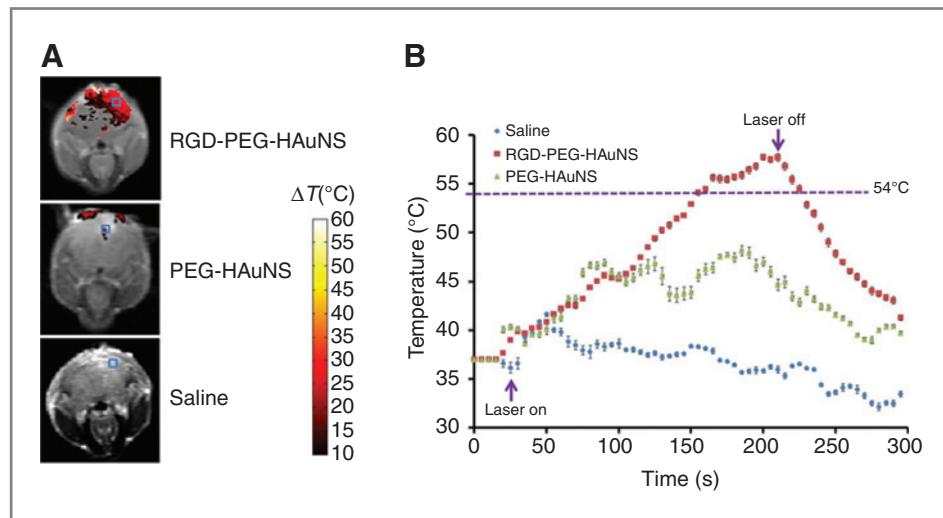


Figure 2. Targeted delivery of  $^{64}\text{Cu}$ -labeled c(KRGDf)-PEG-HAuNS to U87 human glioma in mouse brains. Shown are  $\mu$ PET/CT images (obtained 24 hours after i.v. injection of nanoparticles); photographs of mouse brains stained with hematoxylin and eosin (H&E) and  $\alpha_v\beta_3$  and autoradiographs of mouse brains; and immunofluorescence micrographs of tumor tissue at high magnification. Red,  $\alpha_v\beta_3$ ; green, scattering signal of HAuNS under dark field; blue, 4',6'-diamidino-2-phenylindole (DAPI)-stained cell nuclei; arrows, tumors; bar, 10  $\mu\text{m}$ .

**Figure 3.** Real-time MRTI of U87 human gliomas in mouse brains 24 hours after injection of HAuNS. A, overlap of mouse brain T1-weighted MRI with Magnevist and MRTI at the end of laser irradiation. B, tumor temperature change versus time in the region of interest (blue rectangles in A). Laser (16 W/cm<sup>2</sup> at 808 nm) was applied for 3 minutes between the time points indicated by the arrows.



### PTA therapy with c(KRGDf)-PEG-HAuNS prolongs survival of mice with orthotopic U87 gliomas

Bioluminescence imaging showed significantly decreased luminescence intensity in tumors of mice treated with c(KRGDf)-PEG-HAuNS injection and laser irradiation (Fig. 4A and B). This tumor-ablation effect was observed during the first 9 days after laser treatment (days 9–17), when the luminescence intensity was less than that before laser treatment (day 8). On day 19 and later, the luminescence intensity exceeded that before laser treatment, indicating brain tumor recurrence. In mice treated with PEG-HAuNS plus laser, luminescence intensity in tumors decreased only on day 9. In mice treated with laser alone, c(KRGDf)-PEG-HAuNS alone, or saline, luminescence intensity increased throughout treatment.

The median survival time of mice treated with c(KRGDf)-PEG-HAuNS plus laser (28 days) was significantly more than that of the other groups ( $P < 0.001$ ): PEG-HAuNS plus laser, 19.5 days; laser alone, 17.5 days; c(KRGDf)-PEG-HAuNS alone, 17.5 days; and saline, 16.5 days (Fig. 4C). Histologic examination confirmed that c(KRGDf)-PEG-HAuNS plus laser was the treatment causing the most extensive necrotic response (Fig. 4D). Extensive pyknosis, cytoplasmic acidophilia (10, 17), and corruption of the extracellular matrix of the tumor appeared throughout tumors of mice treated with c(KRGDf)-PEG-HAuNS plus laser. The tumor cells were completely ablated; there were no discernible residue viable tumor cells in the tumor periphery (Fig. 4D, arrow). In mice treated with PEG-HAuNS plus laser or laser alone, approximately 45% and 30% of tumor tissues were necrotized, characterized as pyknosis and medium cytoplasmic acidophilia, and there were large numbers of viable tumor cells in the periphery (Fig. 4D, asterisks). In tumors of mice treated with c(KRGDf)-PEG-HAuNS alone or saline alone, most tumor cells were intact.

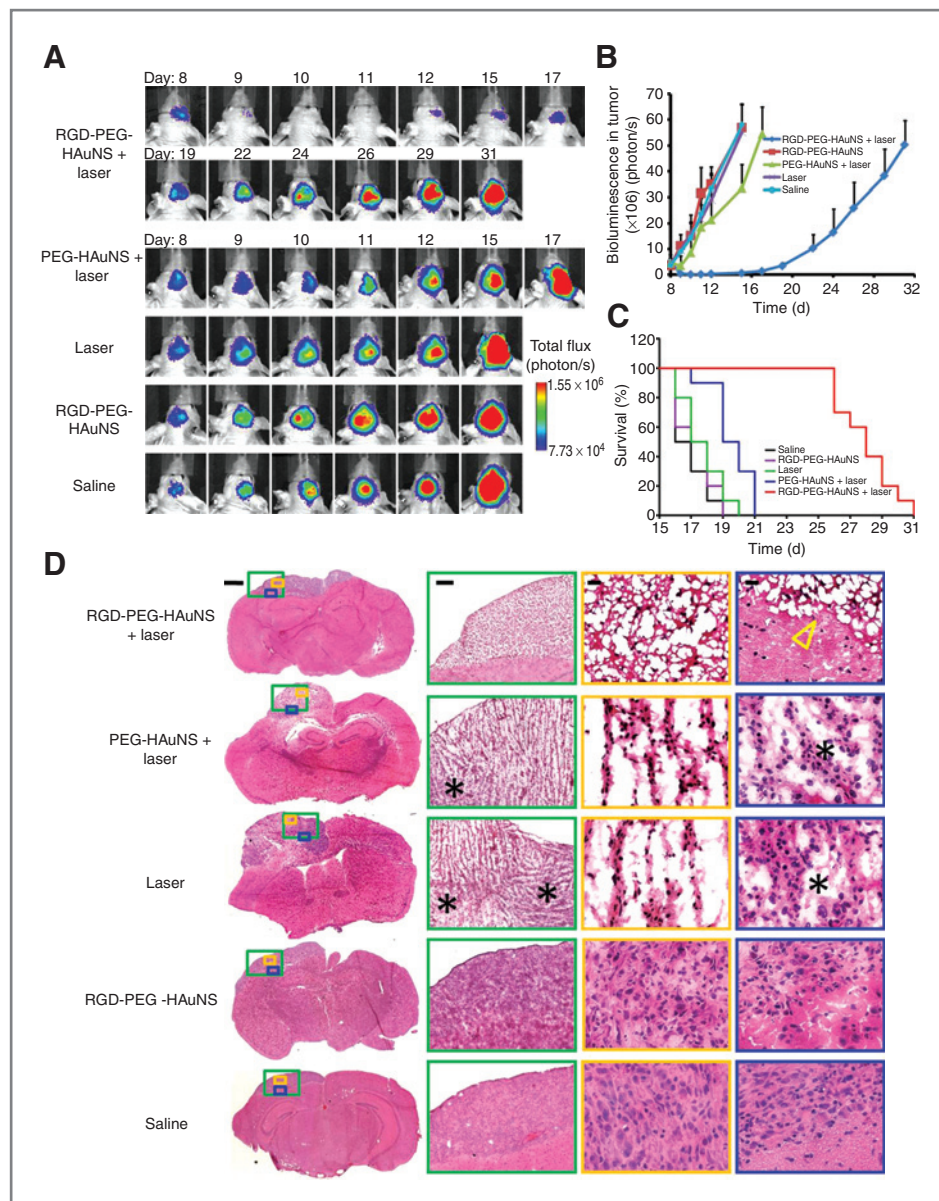
### Discussion

We have shown effective PAT imaging and PTA therapy of glioma mediated by a single preparation of targeted HAuNS on

the basis of their intrinsically high optical absorption cross-section. The findings from this study suggest potential applications of HAuNS as a novel theranostic platform. First, c(KRGDf)-PEG-HAuNS can serve as efficient optical contrast agents for photoacoustic imaging, which may provide cancer diagnosis with high resolution and high sensitivity in addition to conventional imaging modalities. PAT may also be useful for pretreatment diagnosis and real-time monitoring of treatment, as well as assessment of treatment outcome. Second, under the guidance of PAT imaging, accurate and efficient PTA of tumor cells would be instituted simply by switching the laser power from diagnostic level (pulsed laser, 50 mW/cm<sup>2</sup>) to therapeutic level (diode laser, 16 W/cm<sup>2</sup>) on the basis of the high photothermal coupling efficiency of HAuNS. Third, given that  $\mu$ PET/CT using <sup>64</sup>Cu-labeled c(KRGDf)-PEG-HAuNS confirmed selective uptake of the nanoparticles in U87 tumors, PET/CT with <sup>64</sup>Cu-labeled targeted HAuNS should provide an alternative imaging method for HAuNS-mediated PTA therapy.

With these advantages, we envision that for treating an entire tumor volume, delivery of laser energy to the region of interest would be achieved in a way similar to that of interstitial high-dose-rate brachytherapy in that multiple applicators are used to cover the area of interest (18). For individuals with small recurrent tumors, who represent a large and important patient population, this would provide a minimally invasive technique to both diagnose and target the recurrence, using a single fiber. Remaining PAT signal from the tumor bed or beyond after surgery could guide further resection and/or provide opportunity for selective ablation mediated by the targeted HAuNS. If successfully implemented in the clinical setting, our approach described here offers an alternative option over current clinical investigations of glioma theranostics involving CT-guided thermotherapy with superparamagnetic iron oxide nanoparticles in an alternating magnetic field (19, 20). In this case, the nanoparticles are administered intratumorally under neuroanavigational control and pretreatment MRI.

Although no viable tumor cells were found on histologic evaluation after treatment with c(KRGDf)-PEG-HAuNS plus NIR irradiation, we observed regrowth of tumor, suggesting



**Figure 4.** PTA of U87 human gliomas in mouse brains. **A**, representative bioluminescence images of nude mice bearing U87-TGL tumors with different treatments (see Materials and Methods). **B**, quantitative analysis of bioluminescence beginning with treatment administration on day 8 after tumor inoculation ( $n = 5$  per group). **C**, Kaplan-Meier survival curve of tumor-bearing mice treated as in **B** ( $n = 10$  per group). **D**, histologic evaluation of tumor necrosis. Left, representative photographs of tumor-bearing mouse brains stained with hematoxylin and eosin 24 hours after NIR laser irradiation. Right (3 lanes), representative microphotographs at high magnification of the areas corresponding to the boxed areas in the left lane. Arrow indicates no discernible residual viable tumor cells in the tumor periphery. \*, viable tumor cells in mice treated with PEG-HAuNS plus laser or laser alone; bars (left to right), 1 mm, 200  $\mu$ m, 20  $\mu$ m, 20  $\mu$ m.

the presence of residual tumor cells after treatment. Combining PTA therapy and other treatment modalities, such as photothermal chemotherapy made possible with drug-loaded HAuNS or photothermal transfection made possible with HAuNS coated with therapeutic siRNA (14), should lead to further enhanced therapeutic outcome and even cure. Studies toward this goal are currently underway.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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