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CHAPTER V

Novel triple bioluminescence imaging system for monitoring of Glioma response to combined soluble TRAIL and Lanatoside C therapy

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ABSTRACT

We developed a triple reporter system based on Vargula, Gaussia and firefly luciferases for sequential imaging of three different biological processes. We applied this system to monitor the effect of soluble tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL-delivered using an adeno-associated viral AAV vector), in combination with the cardiac glycoside lanatoside C, in different orthotopic glioma models. Binding of sTRAIL on tumor cells activated downstream events leading to an initial decrease in glioma proliferation, followed by tumor re-growth through resistant cells. Co-treatment with lanatoside C sensitized the resistant subpopulation of glioma to sTRAIL-induced apoptosis as monitored by the triple reporter system. Since AAV vectors, TRAIL, and cardiac glycosides have been used in the clinic separately, this therapeutic strategy could be easily adapted to humans. This work is the first demonstration of triple *in vivo* bioluminescence imaging and will have broad applicability in different fields.

INTRODUCTION

Bioluminescence imaging (BLI) using luciferase reporters has been indispensible for non-invasive monitoring of different biological processes in cancer such as tumor volume and transcriptional activation during tumor development/therapy, as well as immune cell infiltration into the tumor environment[1]. The major advantage of using BLI compared to endpoint analysis is that it provides real-time, non-invasive measurements of in situ biological events, giving a complete “picture” of the kinetics of an entire process. Great strides have been made since the seminal study by Contag et al. published in 1995 which was the first demonstration of *in vivo* BLI[2]. For example, as few as 10 cells expressing firefly luciferase can be detected in deep tissue in some animal models[3]. This study followed by the discovery and molecular construction of different luciferases with a multitude of properties including secreted luciferases[4], multi-color light emission spectras for better tissue penetrance *in vivo* and spectral deconvolution[5], increased thermostability[6], and light output (sensitivity)[7]. One limitation to current bioluminescence imaging is that typically only one and at most two luciferase reporters are used to measure one or two parameters[8]. As

tumor formation is a complex process, concurrent measurement of several processes will be important for the development of novel therapeutics and their transition to the clinic.

Tumor necrosis factor apoptosis-inducing ligand (TRAIL) is regarded as a potential anti-cancer agent, however, considerable numbers of cancer cells, especially adult gliomas, are resistant to apoptosis induction by TRAIL[9,10]. Another major disadvantage of using TRAIL for glioma therapy is its inefficient transport across the blood-brain barrier[11]. Through drug screening, we have identified lanatoside C, an FDA approved cardiac glycoside, to sensitizes primary GBM cells to TRAIL-induced apoptosis[12]. Cardiac glycosides including lanatoside C have been recently shown to provide neuroprotection against ischemic stroke showing that these drugs penetrate the brain efficiently[13, 14].

In this study, we explored the use of adeno-associated viral vector (AAV) to deliver soluble TRAIL (sTRAIL) to brain tumors in combination with its sensitizer, lanatoside C for glioma therapy. The AAV vector serotype chosen (AAVrh.8) efficiently transduces the normal brain (primarily neurons) to synthesize and secrete sTRAIL, which in turn binds to death receptors found specifically on glioma cells, and kill them upon lanatoside C treatment. We first developed and optimized triple bioluminescence imaging system using three different luciferases, each specific to its own substrate, from the marine copepod *Gaussia princeps* (Gluc-uses coelenterazine as a substrate), the American firefly *photinus pyralis* (Fluc,-$_D$-luciferin substrate), and ostracod *Vargula* (*Cypridina) hilgendorfii* (Vluc-uses vargulin as a substrate). We then applied this system to monitor sequentially (1) AAV gene delivery; (2) sTRAIL binding to glioma death receptors and activation of downstream events; and (3) tumor response to the combined sTRAIL/lanatoside C therapy. We showed that glioma cells are somewhat resistant to sTRAIL monotherapy and the combined sTRAIL/lanatoside C therapy yields significant delay in tumor growth. This combined therapy could potentially be translated to humans since all its components have been used in clinical trials separately. Further, this study is the first demonstration of triple *in vivo* BLI, which would be applicable to many disease/therapeutic pre-clinical models.

RESULTS

Characterization of a codon-optimized Vargula luciferase for mammalian gene expression. We first cloned the Vluc cDNA, codon-optimized for mammalian gene expression into a lentivirus vector under the control of the CMV promoter (Lenti-Vluc). This vector also expresses the mCherry fluorescent protein under an internal ribosomal entry site (IRES), used to monitor transduction efficiency. Since Vluc cDNA carries a natural signal sequence,[15] it is secreted to the conditioned medium once expressed in mammalian cells. We first compared the level of Vluc secretion by transducing 293T cells with this lentivirus vector and evaluating cell lysates and conditioned medium for Vluc activity using the vargulin substrate. We observed that the majority of Vluc activity (78%) was contained in the medium fraction showing efficient secretion (**Fig. 1a**). Next, we checked the light emission kinetics of Vluc after substrate addition over 5 minutes. We observed a slow decay in light emission with 44% of the initial signal remaining 5 minutes post substrate addition (**Fig. 1b**). To further characterize Vluc as a mammalian cell reporter, we measured the stability of the enzyme over time at 37°C. Conditioned medium from cells expressing Vluc was incubated at 37°C in a humidified cell incubator. Aliquots were taken at different time points and assayed for Vluc activity. We observed that Vluc levels did not drop below initial levels over 12 days indicating high enzyme stability in cells conditioned medium (**Fig. 1c**), similar to that reported for the secreted Gaussia luciferase[16]. We next evaluated Vluc as a reporter to monitor cell viability and proliferation over time. U87 glioma cells transduced with Lenti-Vluc were plated and aliquots of media were collected at different time points. In parallel a commercially available Fluc-based viability assay was performed for comparison. We observed a high correlation (r^2=0.98) between the Vluc viability assay and the established viability assay (**Fig. 1d**).

Figure 1

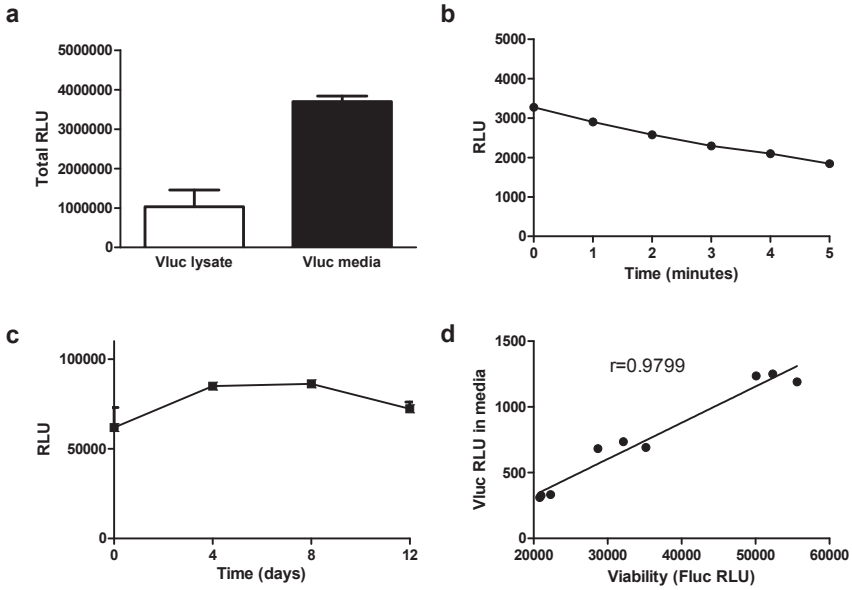

Figure 1. ***In vitro*** **characterization of Vluc-catalyzed luminescence.** (a) A Vluc assay was performed on conditioned medium as well as cell lysates from 293T cells transduced with a lentivirus vector encoding Vluc to determine the fraction (in RLU) of Vluc being secreted. (b) Vluc light emission kinetics was performed by adding vargulin to Vluc-containing media and acquiring signal over time. (c) Stability of Vluc enzyme was determined by assaying an aliquot of Vluc-containing cell-free conditioned medium, incubated at 37 ^{0}C, at different time points. (d) Vluc-based viability assay. 10^5 U87 cells expressing Vluc were seeded in a 12-well plate and an aliquot of conditioned medium was assayed for Vluc activity over time. For comparison, a commercially available Fluc-based viability assay (CellTiter Glo; Promega) was performed.

Optimization of the triple *in vivo* bioluminescence imaging system. In order to validate Vluc as a reporter for *in vivo* imaging, we first characterized its bioluminescence properties in a quantitative tumor model. Nude mice were injected subcutaneously with 2x10^6 U87 cells stably expressing Vluc (through transduction with Lenti-Vluc). Ten days later, mice were injected intravenously (i.v.- through retro-ocular route) with vargulin (4 mg/kg body weight) and imaged using a cooled charge-coupled device (CCD) camera at different time points post-vargulin injection. We observed the peak luminescent signal immediately upon injection of vargulin, which rapidly declined to 25% of starting signal by 6 minutes and down to 10% by 26

minutes (**Fig. 2a**). Next, we compared i.v. versus intraperitoneal (i.p.) injection of the vargulin substrate for Vluc imaging and observed that the peak signal occurred 10 minutes after i.p. injection (**Supplementary Fig. 1**), with the i.v. injection yielding an 8-fold higher photons/min compared to the i.p. injection (**Fig. 2b**). Therefore, the i.v. injection route was used throughout the study.

We then evaluated the potential of Vluc to be multiplexed with Gluc and Fluc for sequential triple bioluminescence imaging by testing luciferase/substrate specificity *in vivo*. U87 stably expressing either Vluc, Gluc or Fluc (under control of the CMV promoter) were implanted subcutaneously in nude mice at three different sites. Ten days later, mice were imaged first for Gluc-mediated bioluminescence imaging by i.v. injection of coelenterazine (5 mg/kg body weight) and acquiring photon counts immediately. Intense luminescence was detected only from tumors expressing Gluc (**Fig. 2c**). Next, mice were imaged after i.v. injection of vargulin (4 mg/kg body weight) and again signal was obtained only from tumor-expressing Vluc. Finally, mice were i.p. injected with *D*-luciferin (150 mg/kg body weight) and imaged 10 min later which showed signal only in tumor-expressing Fluc (**Fig. 2c**). Fluc was imaged last since it is known to have glow-type kinetics in mice. These results show that each of these luciferases is specific to its own substrate and can be multiplexed together in the same biological system to monitor three distinct biological processes sequentially.

Figure 2

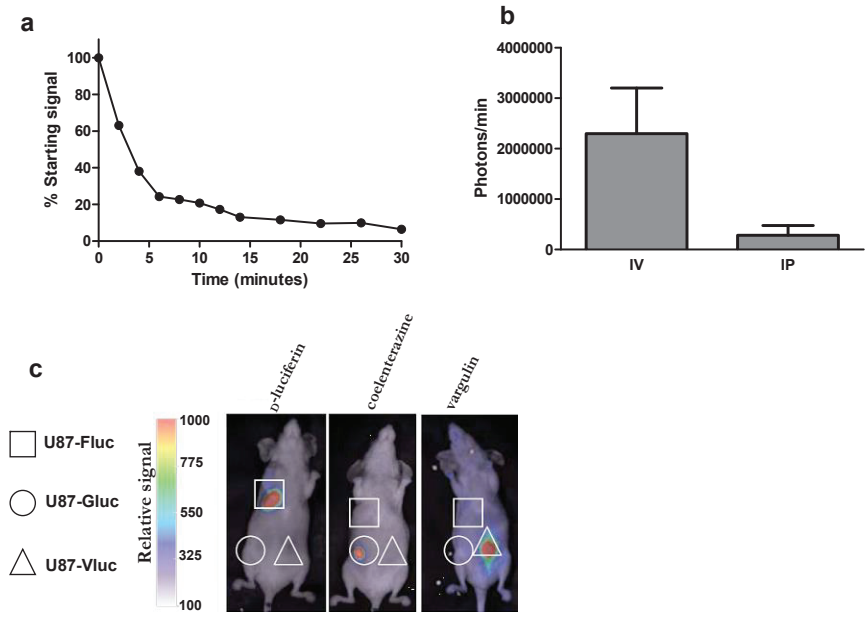

Figure 2. Triple in vivo bioluminescence imaging. (a,b) 2x10^6 U87-Vluc glioma cells were implanted subcutaneously in nude mice. Tumor-associated Vluc bioluminescence signal was imaged after i.v. injection of vargulin over time (a). Tumor associated Vluc signal after i.v. or i.p. injection of vargulin was measured (b). U87 glioma cells stably expressing Gluc, Vluc or Fluc (all under CMV promoter) were injected subcutaneously in nude mice at different sites. Ten days later, Sequential imaging of Gluc, Vluc and Fluc was performed after injection of coelenterazine, vargulin and $_D$-luciferin, respectively (c).

Monitoring of glioma therapy using triple in vivo bioluminescence imaging. We used the triple luciferase reporter system to monitor the effect of glioma to a secreted soluble variant of the anti-cancer agent TRAIL (sTRAIL[17]) in combination with its sensitizer lanatoside C. We first cloned sTRAIL under control of the constitutively active chicken beta-actin (CBA) promoter into an AAV2 ITR-flanked transgene cassette and pseudotyped it with an AAVrh.8 capsid (AAV-sTRAIL; **Fig. 3a).** As a control, we packaged a similar vector expressing GFP driven by the CBA promoter. We chose to use AAVrh.8 as we have previously shown that this serotype yields excellent transduction efficiency of murine normal brain[18]. Injection of AAVrh.8 into the tumor using convection-enhanced delivery results in transduction of primarily

neuronal cells surrounding the tumor[18]. The transduced neurons form a therapeutic zone around the tumor by synthesizing and secreting sTRAIL, which in turn will find and bind its death receptor present specifically on glioma cells. Co-treatment with lanatoside C should allow sensitization of glioma cells to TRAIL's pro-apoptotic effects[12].

To determine the functionality of the AAV-sTRAIL construct, we transduced U87 cells with 10^4 genome copy (g.c.)/cell of AAV-sTRAIL vector or AAV-GFP as a control. Three days later, conditioned media from these cells were harvested and analyzed for TRAIL using an ELISA kit. We observed a TRAIL concentration of 124 ng/ml from conditioned medium of AAV-sTRAIL infected cells while it was undetectable in the media from AAV-GFP control cells, showing the proper expression and secretion of sTRAIL into the conditioned medium of cells**.** Transfer of conditioned media from AAV-sTRAIL transduced cells along with lanatoside C onto U87 cells provided robust cell killing as expected (**Supplementary Fig. 2**).

We next cloned Vluc cDNA under control of CBA promoter in another AAV2 vector pseudotyped with AAVrh.8 capsid (AAV-Vluc; **Fig. 3a**). Vluc was used as a marker for efficient gene transfer. We then engineered U87 glioma cells to stably express Fluc and the mCherry fluorescent protein (U87Fluc) under control of CMV promoter, using a lentivirus vector (**Fig. 3a**). Fluc was used as a marker for tumor volume. Since TRAIL is known to activate a series of events including the nuclear factor kappa B (NF-κB) pathway, we engineered a lentivirus vector expressing Gluc under the control of tandem repeats of NF-κB responsive elements (**Fig. 3a**)[19], and used it to transduce the U87Fluc cells generating U87Fluc/NF-Gluc cells. We initially used U87 glioma cells as a model since these cells are partially resistant to the pro-apoptotic effects of TRAIL[20].

We stereotactically implanted 10^5 U87Fluc/NF-Gluc cells into the striatum of nude mice and allowed tumors to form. Two weeks later, mice were randomly divided into two groups (n=5). The first group was infused into the same tumor implanted site with 10^{10} g.c. of both AAV-Vluc + AAV-GFP (AAV-Vluc/GFP; serving as a negative control for tumor therapy and for imaging of gene delivery) and the second group was infused with 10^{10} g.c. of both AAV-Vluc (to monitor successful transgene delivery) and AAV-sTRAIL (anti-tumor therapy; AAV-Vluc/sTRAIL). As a negative control for Vluc imaging, a group of mice received similar injection of AAV-GFP

alone. Ten days post-vector injection, we monitored AAV gene delivery by injecting (i.v.) mice with vargulin substrate and immediately imaging using a CCD camera. Evident bioluminescent signal (average radiance of $7.5x10^5$ p/s/cm²/sr +/- 2.3E4) was seen at the injection site of all AAV-Vluc injected mice (and not the AAV-GFP control) showing successful gene delivery (**Fig. 3b**). Mice were also imaged at 1, 2, and 3 weeks post-vector injection for tumor-associated Fluc-signal, and therefore tumor size, after i.p. injection of $_D$-luciferin substrate. We observed that AAV-mediated sTRAIL expression slowed the tumor growth as compared to control mice. For example, at week 1 post-vector injection, Fluc imaging showed an average radiance for AAV-Vluc/GFP control mice of 1.03×10^6 photons/s/cm^2/sr while it was only 2.38×10^4 photons/s/cm^2/sr for AAV-Vluc/sTRAIL treated mice (**Fig. 3c,d**). The average bioluminescent signal remained high (1.28×10^6 photons/s/cm^2/sr) for AAV-Vluc/GFP control mice at week 2 and by week 3, 70% of mice were sacrificed due to advanced tumor progression (**Fig. 3c,d**). In contrast, at two weeks post-vector injection, the average signal remained very low (1.8×10^4 photons/s/cm^2/sr) for the AAV-Vluc/sTRAIL injected group (**Fig. 3c,d**). Interestingly, in this group the radiance increased by 214-fold between the two and three week post-vector injection presumably due to gained-resistance to sTRAIL therapy (**Fig. 3c,d**).

TRAIL binding to its death receptors recruits TNFR1-associated death domain protein (TRADD) leading to NF-κB activation[21], we therefore sought to detect NF-κB induction and therefore sTRAIL binding to glioma cells in our model. Mice in both the AAV-Vluc/GFP (control) and AAV-Vluc/sTRAIL groups were imaged 3-4 days post-viral injection for Fluc (tumor size) and for Gluc after retro-orbital injection of coelenterazine. Fluc bioluminescence at the tumor location was detectable in both groups of mice as expected but visible specific Gluc signal detected only in mice injected with AAV-Vluc/sTRAIL and not AAV-Vluc/GFP control animals indicating binding of sTRAIL to glioma cells and induction of the downstream NF-κB pathway (**Fig. 3e**). In two separate experiments, we compared the relative NF-κB induction between the AAV-Vluc/GFP and AAV-Vluc/sTRAIL groups by normalizing the Gluc levels to the tumor size (quantitated by Fluc imaging). In both experiments, we observed a much higher normalized Gluc signal (15-142-fold) in the mice treated with AAV-sTRAIL compared to the control group indicating induction of NF-κB (**Fig. 3f**). These results were reproduced in three independent experiments and shows

that our triple luciferase system could be used to image three independent processes.

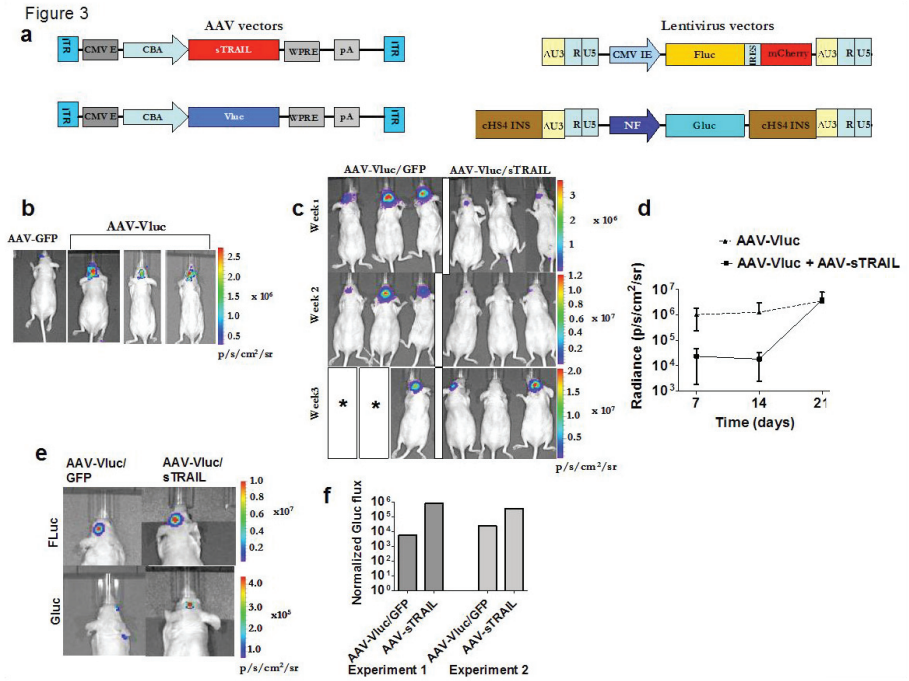

Figure 3. Monitoring of TRAIL-mediated cancer gene therapy with triple bioluminescence imaging. (a) Schematic diagram for the different AAV (left) and lentivirus vector (right) constructs used in this study. AAV vectors: **CMV E**, cytomegalovirus enhancer; **CBA**, chicken beta actin promoter; **WPRE**, woodchuck hepatitis virus posttranscriptional regulatory element; **pA**, bGH poly A signal. Lentivirus vectors: Diagrams shown represent integrated provirus. **CMV IE**, cytomegalovirus immediate early promoter; **cHS4 INS**, chicken β globin hypersensitive site 4 insulator sequence; **NF**, NF-κB inducible promoter. (b-f) 10^5 U87Fluc/NF-Gluc glioma cells were implanted intracranially in nude mice. (b) Two weeks later, the mice brain were infused at the same tumor injection site with 10^{10} g.c. of either AAV-GFP, AAV-Vluc/GFP or AAV-Vluc/sTRAIL (as noted). Vluc bioluminescence imaging was performed 10 days post-vector injection confirming AAV-mediated gene delivery. Representative mice from AAV-Vluc/sTRAIL and AAV-GFP are shown. (c) Tumor volume was monitored by Fluc bioluminescence imaging at different time points. (d) Quantitation of tumor-associated Fluc signal in c. (e) Fluc imaging, a marker for tumor volume, as well as Gluc imaging, a marker for TRAIL binding on glioma cells, was performed. (f) Gluc signal normalized to Fluc signal in (e) to account for tumor size from two separate experiments is shown.

We have previously discovered that a cardiac glycoside, lanatoside C (LanC), sensitizes glioma cells to recombinant TRAIL, thus overcoming their resistance to TRAIL-mediated killing, both in cultured cells and in a subcutaneous model[12]. We then tested the effect of this combined therapy in our orthotopic glioma model using AAV vector to deliver sTRAIL. Nude mice were again implanted into the striatum with 10^5 U87Fluc/NF-Gluc cells as above. Two weeks post-injection (when tumor was formed as monitored by Fluc imaging), mice were randomly divided into two groups, which received injection of either AAV-Vluc/GFP or AAV-Vluc/sTRAIL. Each group was then divided into two subgroups (n=10) which either received i.p. injection of DMSO (vehicle) or LanC (7.5 mg/kg body weight). This resulted in the following four groups: (1) AAV-Vluc/GFP, (2) AAV-Vluc/GFP + LanC, (3) AAV-Vluc/sTRAIL, (4) AAV-Vluc/sTRAIL+LanC. Lanatoside C was injected daily for 3 weeks starting one-day post-vector injection. Gene transfer as well as TRAIL binding to glioma cells was imaged as above, using Vluc and Gluc, which showed similar results to figure 3. Tumor volume was imaged over time with Fluc bioluminescence imaging. At two weeks post-injection, AAV-Vluc/GFP and AAV-Vluc/GFP + LanC treated mice had an average radiances of 1.04×10^6 and 3.6×10^6 photons/s/cm^2/sr, respectively (**Fig. 4a,c**). On the other hand, both AAV-Vluc/sTRAIL and AAV-Vluc/sTRAIL+LanC treated mice had similar radiances to one another, which were significantly lower (>18-fold; $p<0.05$) than AAV-Vluc/GFP and AAV-Vluc/GFP+LanC control groups (**Fig. 4a,c)**. Between 2 and 3 weeks post-vector injection, all mice in the AAV-Vluc/GFP and AAV-Vluc/GFP+LanC were sacrificed due to tumor progression whereas mice from AAV-Vluc/sTRAIL and AAV-Vluc/sTRAIL+LanC remained alive. Imaging of mice in the AAV-Vluc/sTRAIL and AAV-Vluc/sTRAIL+LanC groups at week 3 post-vector injection revealed a 6.7-fold lower mean radiance ($p<0.05$) in the combination treated group as compared to AAV-Vluc/sTRAIL alone showing that LanC had a sensitization effect on glioma cells to sTRAIL when co-delivered (**Fig 4,b-d**).

Figure 4

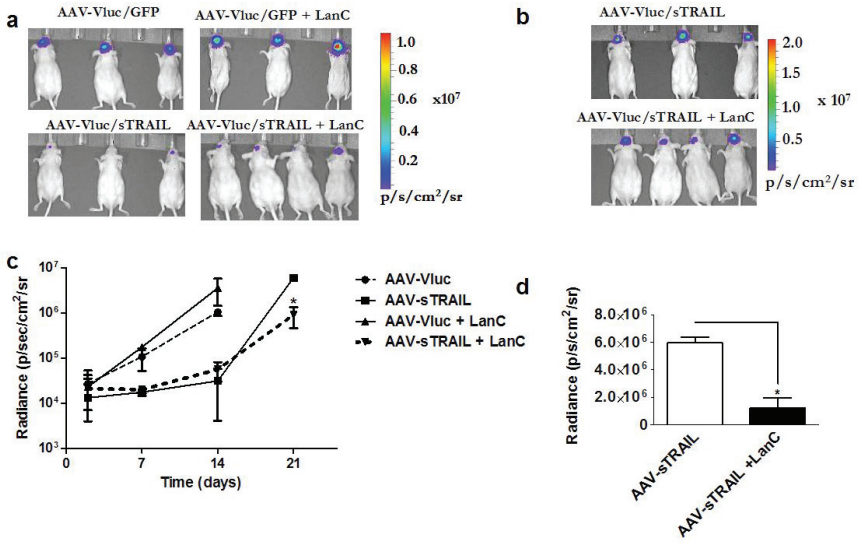

Figure 4. Lanatoside C enhances TRAIL-mediated therapy against intracranial gliomas. Mice bearing U87Fluc/NF-Gluc gliomas received an infusion of AAV-Vluc/GFP or AAV-Vluc/sTRAIL. Each group was divided into 2 subgroups, which either received vehicle or LanC. Tumor growth was monitored over time by Fluc bioluminescence imaging. (a,b) Bioluminescence images from representative mice in each group at week 2 (a) and 3 (b). (c) Quantitation of tumor-associated bioluminescence of the different treatment groups over time. (d) Quantitation of Fluc signal in the AAV-Vluc/sTRAIL and AAV-Vluc/sTRAIL+LanC groups at week 3. Mice in AAV-Vluc/GFP and AAV-Vluc/GFP+LanC had been sacrificed prior to week 3 imaging due to tumor progression.

To check whether lanatoside C could re-sensitize TRAIL-resistant cell population to its apoptotic effects, mice bearing established U87Fluc/NF-Gluc were injected with AAV-Vluc/sTRAIL as above. Sixteen days post-vector injection, mice were treated for 6 consecutive days with LanC. At day 22, Fluc imaging revealed a 17-fold decrease in tumor volume confirming sensitization (**Fig. 5a)**. These mice were then left off the drug for 7 days. At day 29, Fluc imaging revealed a 60-fold increase in tumor-associated signal indicating that U87 glioma cells regained TRAIL resistance in the absence of LanC (**Fig. 5a**). Placing the mice back on this drug from day 29 to day 36 again showed re-sensitization of glioma tumors to TRAIL (**Fig. 5a**). These data suggest that LanC could re-sensitize resistant glioma cells to TRAIL-induced cell death. In another group of mice, we allowed the glioma cells to gain resistance to TRAIL before treating with one cycle of LanC (given at day 29). U87Fluc/5NF-Gluc

were implanted in the brain of mice, injected with AAV-Vluc/sTRAIL or AAV-Vluc/GFP as above and imaged for tumor-associated Fluc signal over time, which showed tumor re-growth. Fluc imaging at day 36 showed a 1.6-fold decrease in Fluc signal and therefore sensitization of resistant glioma cells to TRAIL in the AAV-Vluc/sTRAIL injected group (**Fig. 5b**). On the other hand, the control group injected with AAV-Vluc/GFP+LanC showed a 6.3-fold-increase in tumor volume between day 29 and 36 (**Fig. 5c**), proving that the observed anti-tumor effect is due to the combined sTRAIL and LanC Therapy.

Figure 5

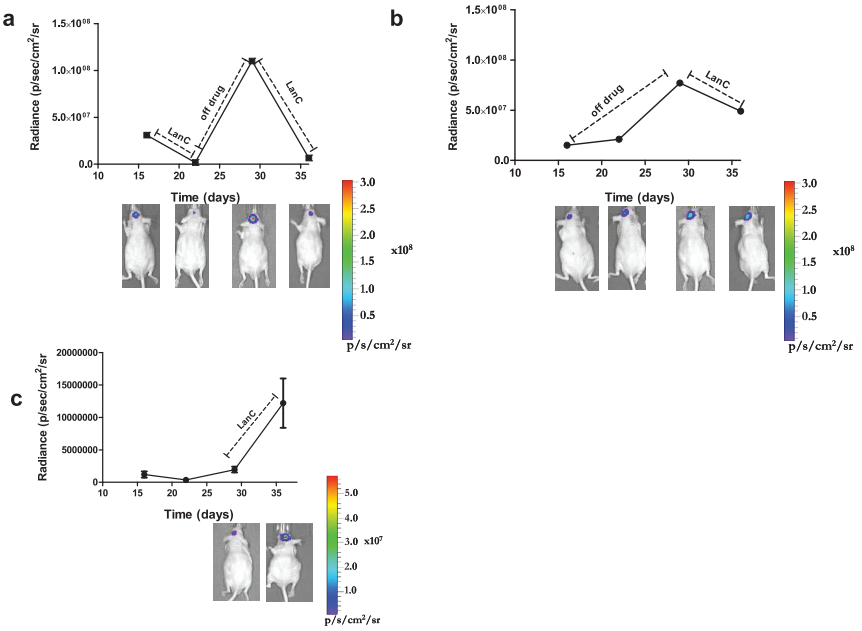

Figure 5. Lanatoside C re-sensitizes glioma tumors to TRAIL. (a) Mice brains bearing U87Fluc/NF-Gluc gliomas were infused with AAV-Vluc/sTRAIL. Sixteen days later, mice were treated with two 6-days cycle of LanC with a 7 days period off drug between each cycle. Tumor volume was monitored by Fluc bioluminescence imaging. (b) Similar treatment as (a) except mice received only one cycle of LanC treatment 30 days post-vector injection. (c) Control experiment in which mice bearing U87Fluc/NF-Gluc gliomas were injected with AAV-Vluc/GFP vectors and then subjected to one cycle of LanC treatment. Tumor volume was imaged at the beginning and end of drug treatment. Representative mouse from each group is shown under each time point.

We next evaluated our AAV-sTRAIL+LanC therapy in an invasive primary glioma orthotopic xenograft model using human GBM8 tumor stem-like cells[22] stably expressing Fluc. These cells are cultured as neurospheres and we observed that they are semi-sensitive to monotherapy of TRAIL and LanC, However, combined therapy induces >95% cell death (**Supplementary Fig. 3**). GBM8-Fluc cells were implanted into the striatum of nude mice. At day 14 post-tumor injection, mice were imaged for Fluc signal (tumor volume) and then divided into 4 different groups (n=8), such that the mean bioluminescence of each group was similar to one another. At day 22, when imaging showed no significant difference in tumor volume between groups (p=0.9955, **Supplementary Fig. 4**), each group of mice was treated as follows: (1) AAV-Vluc/GFP, (2) AAV-Vluc/GFP+LanC, (3) AAV-Vluc/sTRAIL, (4) AAV-Vluc/sTRAIL+LanC. Tumor volume was monitored once/week using Fluc BLI over 4 weeks. One week post-treatment, the Fluc signal in the AAV-Vluc/GFP and AAV-Vluc/GFP+LanC group increased by 8.5-fold and 17-fold, respectively (Fig. 6a). In contrast, groups of mice treated with AAV-Vluc/sTRAIL or AAV-Vluc/sTRAIL+LanC showed a 4-fold and 7.2-fold decrease in Fluc signal, and therefore tumor volume, respectively (**Fig. 6a**). The AAV-Vluc/GFP and AAV-Vluc/GFP+LanC groups reached a maximum signal between 2 and 3 weeks post-treatment. Mice treated with AAV-Vluc/sTRAIL continued to have a lower signal compared to the control group, although the signal increased 20-fold between weeks 1-4 (**Fig. 6a**). On the other hand, mice in the co-treatment group which received AAV-Vluc/sTRAIL+LanC group only increased 1.6-fold between weeks 1-4 (**Fig. 6a**). Histological analysis of brains from mice in the different treatment groups was performed at the time of sacrifice. Mice treated with the control AAV-Vluc/GFP vector showed large multi-lobed tumors that were easily visualized by Hematoxylin and Eosin (H&E) stain (**Fig. 6b**). Large tumors at the injected side were also apparent in the control AAV-Vluc/GFP+LanC treated mice (**Fig. 6b**). In contrast, mice treated with AAV-Vluc/sTRAIL had more diffuse tumor with “ring like” phenotype around the site of vector injection (striatum), with secondary tumor masses away from injection site, and tumor cell infiltration along the corpus callosum (**Fig. 6b,c**). In mice treated with both AAV-Vluc/sTRAIL+LanC, no visible tumor masses were observed at the site of vector injection showing efficient therapy of the primary tumor mass (**Fig. 6b**). However, tumor cells migration away from the therapeutic zone was also observed (**Fig. 6c**).

Figure 6

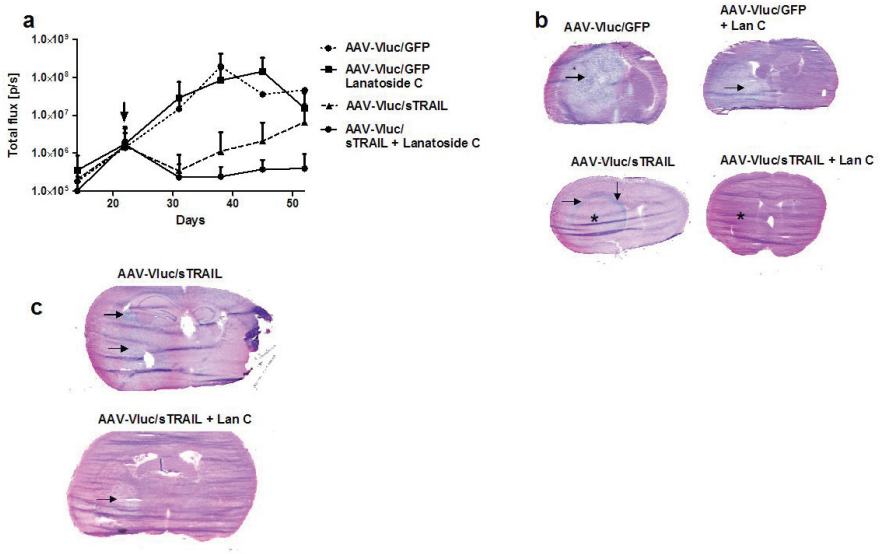

Figure 6. Combined AAV-sTRAIL and lanatoside C therapy slows invasive primary glioma growth. (a) Mice were stereotactically injected with human GBM8 tumor stem-like cells stably expressing Fluc. Three weeks later, mice were divided into 4 different treatment groups as noted. Fluc imaging for tumor size was performed over time. Arrow indicates the start of the treatment. (b-c) H&E histological analysis of a representative mouse from each group at the site of vector injection (b) and distal from the tumor/vector injection site (c) are shown. * indicates tumor/vector injection site. Arrows indicate tumor cells migration away from injection site.

DISCUSSION

Luciferases have played a major role in advancing our understanding of biological processes. A broader array of biocompatible, non-toxic and efficiently- expressed reporters that can be used together with existing luciferases can serve to expand this potential. The present study demonstrates for the first time a triple luciferase reporter system suitable for *in vivo* bioluminescence imaging. We showed that *Vargula* luciferase can be multiplexed together with *Gaussia* and firefly luciferases for sequential monitoring of three distinct biological phenomena. We also showed that this triple bioluminescence imaging yield specific, detectable bioluminescence signal

deep within the brain (with intact skull, ~3.5 mm) of mice. Importantly, we successfully employed this reporter system to monitor three different biological processes in an orthotopic brain tumor model, thus validating the system for different applications.

Since the cloning and sequencing of *Vargula* (formerly *Cypridina*) *hilgendorfii* (Vluc) cDNA in 1989[15], several reports have demonstrated the usefulness of this enzyme for luminescence assays[23-26]. However, to our knowledge, no reports have applied it for *in vivo* imaging probably due to the unavailability of its substrate, vargulin, which is now commercially available. Vluc cDNA possess a signal sequence and therefore is naturally secreted from cells allowing real time, multi-time point analysis from the same cells[23, 27]. In this study, we characterized a codon-optimized Vluc cDNA for mammalian gene expression and showed Vluc to be very stable with no significant decline in activity over 12 days in cell-free conditioned media at 37 °C. Despite the fact that the majority of Vluc was found in the conditioned media of mammalian cells, the intracellular Vluc level was efficient for *in vivo* imaging. In applications where higher sensitivity is required, a membrane-bound variant of Vluc[27] could be used which should yield higher cellular activity similar to a recent report for *Gaussia* luciferase[28, 29].

Systems for multi-modal imaging exist which incorporate different technologies such as fluorescence, bioluminescence, positron emission tomography (PET), and magnetic resonance imaging (MRI)[30]. While substantial multi-parameter information can be gained by these systems, they have several drawbacks including high cost (e.g. MRI), logistical concerns such as short half-life of some PET probes, and broad technical expertise. While fluorescent reporter-based imaging as well as luciferases emitting at different wavelengths can be used for multi-imaging applications, they require expensive and complicated instrumentation with spectral deconvolution for visualizing of each biological parameter of interest[31,32]. Furthermore, fluorescence-based imaging is limited by a high background due to tissue autofluorescence as well as single animal analysis. On the other hand, BLI has low background noise and is thus more sensitive for deep tissue imaging applications[1]. The triple *in vivo* bioluminescence imaging system described here yields multi-parameter information distinguished by sequential imaging using different specific substrates, while remaining both cost effective and highly sensitive, as well as being user friendly

since only a simple CCD camera is required with no need for sophisticated instrument hardware/software. This same system can be extended and applied to any field to monitor three distinct biological phenomena, allowing one to obtain maximal information in animal models

TRAIL has been regarded as an anti-cancer agent, however, significant cancer types, including gliomas, are resistant to TRAIL-induced cell death[9]. Mechanisms of resistance include downregulation of death receptors[33], decoy receptor expression[34], as well as overexpression of the caspase-8 inhibitor, c-FLIP, due to deregulation of the mTOR signaling pathway[35]. Additionally NF-κB induction by TRAIL has been reported as a tumor cell resistance mechanism[10,36]. Through drug screening, we have previously shown that the family of cardiac glycosides, including lanatoside C, sensitizes glioma cells to TRAIL-induced cell death partially through upregulation of death receptors[12,37]. Cardiac glycosides have been recently shown to provide neuroprotection against ischemic stroke[13,14] and therefore penetrate the brain efficiently. Another disadvantage of using TRAIL for brain tumor therapy is its inability to cross the blood-brain barrier. In this study, we circumvented this problem by delivering sTRAIL directly to brain tumor environment by AAV-mediated gene delivery. By engineering the normal brain to synthesize and secrete sTRAIL, it can form a zone of resistance against newly developed glioma, which can be treated with lanatoside C therapy. Since TRAIL, AAV vectors, and lanatoside C have been used separately in clinical trials, this therapeutic approach can be potentially translated to humans. In addition to the more traditional U87 glioma model in which sTRAIL and LanC showed a therapeutic benefit, we tested this therapy against an invasive human glioma model, GBM8[22]. While AAV-sTRAIL showed a therapeutic response compared to the controls, mice eventually succumbed to tumor burden. H&E staining revealed a ring-like tumor formation around the vector injection site (highest concentration of sTRAIL), as well as secondary tumor formation away from injection site due to tumor cell migration. On the other hand, the combined AAV-sTRAIL+LanC group showed significantly lower bioluminescent signal over the course of treatment, as compared to AAV-sTRAIL alone, with practically no tumor at the injection site as shown by H&E staining. However, tumor cell migration away from the injection site was also observed which eventually lead to animal death. To enhance this combined therapy against invasive tumor cells, a multi-injection

approach of the vector may be required. Alternatively, systemic injection of an AAV vector which can lead to global gene delivery (e.g. AAV9) may allow for widespread sTRAIL expression in the brain[38].

METHODS

Cells: U87 human glioblastoma cell line and 293T human kidney fibroblast cells were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO) and 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) in a humidified atmosphere supplemented with 5% CO2 at 37°C. GBM8 tumor stem-like cells were grown as neurospheres as previously described[22]. Briefly, spheres were cultured in Neurobasal medium (Invitrogen) supplemented with 3mM L-Glutamine (Mediatech), 1x B27 supplement (Gibco), 0.5x N2 supplement (Gibco), 2 µg/ml heparin (Sigma), 20 ng/ml recombinant human EGF (R & D systems), 20 ng/ml recombinant human FGF2 (Peprotech) and 0.5x penicillin G/streptomycin sulfate/amphotericin B complex (Mediatech).

Adeno-associated viral vectors and lentivirus vectors. The AAV-Vluc vector was constructed by replacing EGFP in AAV-CBA-EGFP[39] with the human codon optimized Vluc cDNA (a kind gift from Dr. Rampyari Walia, Targeting Systems, El Cajon, CA). AAV-sTRAIL vector consists of a transgene cassette for soluble, secreted TRAIL carrying amino acid (a.a.) 1-150 from human Flt3L, an isoleucine zipper domain, and the extracellular domain (a.a. 114-281) of the human TRAIL designed based on previously reported h-Flex-zipper-TRAIL[17]. In all these AAV vectors, gene expression is controlled by a hybrid cytomegalovirus enhancer/chicken beta actin promoter (CBA). All vectors carry a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) downstream of the transgene. AAV vector pseudotyped with AAVrh.8 capsids were produced by co-transfection of 293T cells using calcium phosphate precipitation of vector plasmid, a mini-adenovirus helper plasmid pFΔ6 (from Dr. Weidong Xiao, Univ. Penn. Med. Ctr., Philadelphia, PA), and AAVrh.8 helper plasmid pAR8 as described[39]. AAV vectors were purified and titered, as described[39], yielding typical titers of 10^{13} g.c. per ml[18]. For sTRAIL

expression in culture, AAV vectors were packaged as AAV2 since this serotype is known to transduce cells in culture much more efficiently as compared to AAV2 genome pseudotyped with AAVrh.8.

U87 cells stably expressing Fluc and mCherry were generated by transduction of these cells with CSCW2-Fluc-ImCherry lentivirus vector at a multiplicity of infection of 10 transducing units/cell as described[18]. These cells were subsequently transduced with Lenti-NF-Gluc, a lentivirus vector encoding a Gluc under the control of 5 tandem repeats of NF-κB-responsive elements[19]. These double-transduced U87 cells are referred to as U87Fluc/NF-Gluc cells. The lentivirus vector encoding Vluc was constructed by replacing the Fluc insert in CSCW2-Fluc-ImCherry with the human codon-optimized Vluc cDNA. The lentivirus vector expressing Gluc was previously described[16]. U87 cell lines stably expressing Vluc or Gluc were generated in a similar manner to Fluc. GBM8 cells stably expressing Fluc and mCherry were generated by transduction of these cells with CSCW2-Fluc-ImCherry as above.

In vivo tumor models. All animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care following guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Six-eight weeks athymic nude mice were anesthetized with a mixture of Ketamine (100 mg/kg) and Xylazine (5 mg/kg) in 0.9% sterile saline. For subcutaneous tumors, mice were injected with 100 μL of a 50:50 mixture of Matrigel™ Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ) and 1-2 million U87 cells expressing either Gluc, Vluc or Fluc resuspended in Opti-MEM. For the brain tumor model, 10^5 U87Fluc/5NF-Gluc cells (in 2 μL Opti-MEM) were intracranially injected in the left midstriatum of nude mice using the following coordinates from bregma in mm: anterior-posterior +0.5, medio-lateral +2.0, dorso-ventral −2.5. These injections were performed using a Micro 4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, FL) attached to a Hamilton syringe with a 33-gauge needle (Hamilton, Rena, NV) at a rate of 0.2 μL/min. For implantation of GBM8-Fluc spheres in mice, cells were dissociated with Accutase (Innovative Cell Technologies, San Diego, CA) 2 days before injection. On the day of injection the majority of spheres were of medium size of approximately 100 cells/sphere, which were left intact for implantation. Approximately $3x10^5$ cells (3000

spheres) were injected per mouse in a volume of 3 µl at 0.3 µl/min using the microsyringe pump and a 33-gauge needle as above.
For AAV vector injection, mice were anesthetized as above and injected intracranially using the same coordinates as for tumor injections. AAV vectors were infused at a rate of 0.2 µL/min using a Micro 4 Microsyringe Pump Controller attached to a Hamilton syringe with a 33-gauge needle.

In vitro and in vivo luciferase imaging. *D*-luciferin was purchased from Gold Biotechnology® (St. Louis, MO) and resuspended at 25 mg/ml in PBS. For Fluc imaging, mice were injected i.p. with 200 mg/kg body weight of *D*-luciferin and imaging was performed 10 min later. Coelenterazine was obtained from NanoLight™ Technology (Pinetop, AZ) and resuspended at 5 mg/ml in acidified methanol. For Gluc imaging, mice were injected i.v. (through retro-orbital sinus) with 5 mg/kg body weight of coelenterazine solution diluted in PBS and imaging was performed immediately. Vargulin substrate (*Cypridina* luciferin) was obtained from NanoLight™ Technology and was resuspended at 5 mg/ml in acidified methanol or from Targeting Systems, sold as a solution. For Vluc as a marker for cell proliferation, and since Vluc was shown to be stable in the conditioned medium, we refreshed the media of cells 4 hours before measurements to avoid accumulation of the reporter. For Vluc *in vivo* imaging, mice were injected i.v. (unless otherwise noted) with 4 mg/kg body weight (diluted in PBS) and imaging was performed immediately. Imaging was performed using an IVIS® Spectrum optical imaging system fitted with an XGI-8 Gas Anesthesia System (Caliper Life Sciences, Hopkinton, MA). Bioluminescent images were acquired using the auto-exposure function. Data analysis for signal intensities and image comparisons were performed using Living Image® software (Caliper Life Sciences).

TRAIL ELISA. The functionality of the AAV-sTRAIL vector was tested by transducing U87 cells with 10^4 g.c./cell with AAV2-sTRAIL or a negative control vector AAV2-GFP. Three days later, media was harvested from all wells and a Quantikine Human TRAIL ELISA (R&D Systems, Minneapolis, MN) was performed per manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.01 software (LaJolla, CA). For comparisons between two samples, an unpaired two-tailed t test was performed. A p value of < 0.05 was considered to be statistically significant. For analysis between multiple groups, a one-way analysis of variance (ANOVA) was performed followed by a Bonferroni's Multiple Comparison Test to compare 2 groups.

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