Functional Drug Screening Assay Reveals Potential Glioma Therapeutics

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ABSTRACT

Here we describe a novel functional screening assay based on bioluminescence monitoring of the naturally secreted *Gaussia* luciferase (Gluc) in the conditioned medium of cultured cells. Using this assay, we identified small-molecule drugs that sensitized brain tumor cells to the tumor necrosis factor-related apoptosis-inducing ligand-induced cell death. Human glioblastoma multiforme cells were engineered by gene transfer to express Gluc as a reporter for cell viability, which can be monitored over time by bioluminescence measurements using a plate luminometer. We have optimized the Gluc assay for screening and validated it using the National Institute of Neurological Disorders and Stroke (NINDS) custom collection II library consisting of 1,040 drugs and bioactive compounds, most of which are Food and Drug Administration-approved and are able to cross the blood-brain barrier. We found that the cardiac glycosides family sensitized glioblastoma multiforme cells to the tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. In conclusion, the Gluc secretion assay is a robust tool for functional drug screening and can be applied to many different fields including cancer.

INTRODUCTION

Cell-based high-throughput screening (HTS) assays are commonly used for drug discovery and provide a valuable tool for identification of novel cancer therapeutics. For an assay to be suitable for HTS, it preferably needs to be facile and cost-effective with short reaction time and should be without the

need of sample processing before measurement. Fluorescence- and bioluminescence-based assays have been shown to be useful tools for HTS.¹⁻⁴ The bioluminescence-based assays rely on the use of a luciferase as a reporter for cell viability.¹ Luciferases encompass a number of enzymes that catalyze light-producing chemical reactions in living organisms by using molecular oxygen to oxidize their substrate luciferin. Previously, we characterized a luciferase from the marine copepod *Gaussia princeps* (Gluc, 185 aa, 19.9 kDa), which is the smallest luciferase known, naturally secreted and emitting light at a peak of 480 nm.⁵ We also showed that Gluc is over 2,000-fold more sensitive than Firefly luciferase (Fluc) and Renilla luciferase (Rluc), and over 20,000-fold more sensitive than the secreted alkaline phosphatase in mammalian cells.^{5,6} As Gluc is naturally secreted and does not require ATP for bioluminescence activity, it can be reported by viable cells themselves as well as their immediate environment.

The sensitive and relatively quick read-out of photon emission allows screening for thousands of molecules in a labor-, time-, and cost-effective manner. Although fluorescence assays are more established for HTS, the wide dynamic range as well as the high sensitivity of bioluminescence may offer a better alternative for cell-based drug screens.⁷ One straightforward application of luciferases in drug screening is their use as cell viability markers. This technique is useful particularly for oncology drugs in order to identify molecules with specific toxicity toward tumor cells.⁸

Tumor necrosis factor apoptosis-inducing ligand (TRAIL) is regarded as a potential anticancer agent; however, considerable number of tumor cells including glioblastoma multiforme (GBM) are resistant to it.⁹⁻¹¹ In this study, we have optimized the Gluc assay for high-throughput drug screening to find drugs that sensitize GBM cells to TRAIL. This secreted reporter was used in a cell-based drug screening assay with high sensitivity and sufficient temporal resolution for assaying cell viability in a 96-well plate format and, more importantly, to study the kinetics of the drug action over time. This drug screening revealed the cardiac glycosides family as potent TRAIL sensitizers for glioblastoma cells.

MATERIALS AND METHODS

Cell Culture

U87 human glioma cells and HEK 293T human fibroblast cells were obtained from the American Type Tissue Collection. Gli36

ABBREVIATIONS: CFP, cyan fluorescent protein; CMV, cytomegalovirus; CV, coefficient of variation; DMSO, dimethyl sulfoxide; Fluc, Firefly luciferase; GBM, glioblastoma multiforme; Gluc, *Gaussia* luciferase; HTS, high-throughput screening; IC₅₀, median inhibition concentration; IRES, internal ribosome entry site; NFκB, nuclear factor-κB; NINDS, National Institute of Neurological Disorders and Stroke; RLU, relative light unit; PBS, phosphate-buffered saline; RT, room temperature; SD, standard deviation; TRAIL, tumor necrosis factor apoptosis-inducing ligand.

human glioma cells were obtained from Dr. Anthony Capanogni, University of California, Los Angeles (UCLA), CA. Primary GBM cells were dissociated from tumor sections of one glioblastoma patient at the Massachusetts General Hospital, under the institutional review board approval for discarded tissues. Portions of GBMs at the time of resection were immediately placed in oxygenated artificial cerebrospinal fluid (124 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 2 mM CaCl₂, 10 mM D-glucose, 26 mM NaHCO₃, pH 8) on ice. The dissociation of the tumor and cell isolation process was initiated within 30 min after removal by surgery. Around 10–15 million primary cells were obtained from each piece of tumor. These cells were then transduced with the lentivirus vector carrying the expression cassette for Gluc and the cyan fluorescent protein (CFP) by adding the vector directly to these cells (Multiplicity of Infection [MOI] = 30–50) and monitoring transduction efficiency by CFP fluorescence microscopy. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 U penicillin, and 0.1 mg/mL streptomycin (Sigma), at 37°C in a 5% CO₂ humidified incubator.

Gluc Lentivirus Vector Construction

Gluc was cloned in CSCW, a self-inactivating lentivirus vector, under the control of the cytomegalovirus (CMV) immediate early promoter.¹² This promoter also controls the expression of CFP separated from Gluc by an internal ribosome entry site (IRES). This construct referred to as pCSCW-Gluc-IRES-CFP was packaged into lentivirus vectors as described.¹² Briefly, 293T cells were cotransfected with the pCSCW-Gluc-IRES-CFP plasmid, the lentivirus packaging genome CMV Δ 8.91 (provided by Dr. Didier Trono, University of Geneva, Switzerland), and envelope-coding plasmid (pVSV-G; provided by Dr. Miguel Sena-Estevés, Massachusetts General Hospital). The supernatant containing the lentivirus vectors was harvested after 48 and 72 h and titered as transducing units/mL on 293T cells in the presence of 10 μ g/mL Polybrene[®] (Sigma) by counting the CFP-positive cells at 48 h postinfection. Typical obtained titers were 10⁸ transducing units/mL.

Small-Molecule Library and Functional Drug Screening

The NINDS Custom Collection small-molecule library comprises 1,040 active compounds dissolved in dimethyl sulfoxide (DMSO, 10 mM), which were compiled by MicroSource Discovery Systems. A working stock of this library was generated by diluting those compounds to a final concentration of 1 mM in DMSO. Recombinant human TRAIL used is the 19.6-kDa protein comprising the full-length of the tumor necrosis factor-like extracellular domain of TRAIL (GF092; Chemicon International; Temecula, CA). Cells were plated in a 96-well plate (3,000 cells/well) in the presence or absence of TRAIL (50 ng/mL). Immediately after plating of the cells, the drugs from the 1 mM working stock were added to a final drug concentration of 1 μ M and 0.1% DMSO (v/v). In each plate, the first row was treated with 0.1% DMSO and the last row with TRAIL alone serving as controls. The results were analyzed using Excel by exporting the data to a spreadsheet in which the data were presented as % RLU of Gluc

expression with the DMSO-treated wells set at 100%. The values from the TRAIL/drug-treated plates were then compared with the drug-treated plates and an automatic FAIL or PASS signal was generated, in which FAIL denotes a potential hit. The different steps of the drug screening assay used in this study are summarized in Table 1.

Luciferase Activity Measurements

All experiments were performed in 96-well plates, with cell density of 1,000–3,000 cells/well. Aliquots of the cell-free conditioned medium (20 μ L) were subjected to Gluc measurement by adding different concentrations of coelenterazine (ProLume/Nanolight, Pinetop, AZ; dissolved in methanol [stock solution] and further diluted in phosphate-buffered saline [PBS; work solution]) and acquiring photon counts using a luminometer (Dynex, Richfield, MN). The signal was measured for 4 s and integrated over 2 s.

ATP-Based Cell Viability Assay

Cell viability was analyzed using Cell Titer-Glo[®] (Promega, Madison, WI), a bioluminescent assay that estimates the cell number based on ATP quantification and firefly luciferase. This assay was performed as recommended by the manufacturer.

Statistical Analysis

The robustness of the Gluc screening assay was determined by calculating the *Z'* factor as follows: *Z'* factor = $1 - [3(\text{SD}_{\text{Gli36}} + \text{SD}_{\text{Gli36-Gluc}})/(\mu_{\text{Gli36}} - \mu_{\text{Gli36-Gluc}})]$.¹³ In addition, the coefficient of variation (CV) was measured using the formula: CV = SD/ μ , aiming at a CV of <10%, including the variation in handling, substrate stability, luminometer injection, and measurement errors. IC₅₀ determination was performed based on nonlinear curve fitting using GraphPad Prism 5.0. Significance levels (Student's *t*-test) were calculated using standard Microsoft Excel spreadsheets.

RESULTS

Gluc-Based Cell Viability

First, we developed a functional cell-based screening assay based on the naturally secreted Gluc. An expression cassette for Gluc and CFP,¹⁴ separated by an internal ribosomal entry site element under the control of a CMV promoter, was cloned into a lentivirus vector (Fig. 1A). CFP was used as a mean to determine the titer of the lentivirus vector and to easily visualize the transduction efficiency, which is usually >90% in glioblastoma cells (Fig. 1B). Upon transduction, Gluc is first expressed in the cells and then processed through the secretory pathway, followed by its release into the conditioned medium.⁶ To evaluate whether Gluc expression and secretion is linear with respect to cell number, Gli36 human glioma cells expressing Gluc and CFP (Gli36-Gluc-CFP) were plated in a 96-well plate in a range of 100–100,000 cells per well. After 24 h, Gluc bioluminescent activity was measured in an aliquot of the conditioned medium. As few as 10 cells could be detected with the Gluc assay in the conditioned medium, with the Gluc signal being linearly related to the cell number in a range covering over 5 orders of magnitude (Fig. 1C). Further, to determine whether Gluc expression

Table 1. Protocol Table for the *Gaussia* Luciferase Functional Screening Assay

Step	Parameter	Values	Description
1	Transduce cells	MOI: 30–50	Transduce glioma cells with lentivirus vector expressing Gluc and cyan fluorescent protein
2	Plate cells	100 μ L final volume	Plate U87-Gluc-CFP (3,000 cell/well)
3	Dilute library compounds	1 mM final concentration	Use DMSO as a diluent
4	Add compounds	1 μ M final concentration	Add 1 μ L of each compound per well
5	Collect aliquots	20 μ L/well	Transfer 20 μ L aliquots of the cells conditioned medium to a white or black plate
6	Assay read-out	1.2 μ M coelenterazine	Perform the Gluc assay by injecting coelenterazine into each well and acquiring photon counts using a luminometer
7	Data analysis		Use Excel spreadsheets

Step Notes

- Cells were plated in 96-well plates. For the plates treated with TRAIL, the latter was added directly into the cell culture medium during cell plating at a final concentration of 50 ng/mL.
 - Dilutions were performed in 96-well plates using a multichannel pipette.
 - In plates treated with the compound only (no TRAIL), the cells in columns 1 and 12 were treated with 0.1% DMSO, whereas in plates treated with compounds + TRAIL, cells in column 1 were treated with 0.1% DMSO and in column 12 with TRAIL at 50 ng/mL.
 - Aliquots can be used directly to measure Gluc activity or stored at $<4^{\circ}\text{C}$.
 - Coelenterazine diluted in phosphate-buffered saline, preincubated at room temperature for 30 min, and automatically injected using a 96-well plate luminometer. The signal was measured for 4 s and integrated over 2 s.
 - The data were presented as % Relative Light Unit (RLU) of Gluc expression where the DMSO-treated wells are set to 100%.
- Gluc, *Gaussia* luciferase; DMSO, dimethyl sulfoxide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CFP, cyan fluorescent protein.

could be used to monitor cell growth and proliferation, Gli36-Gluc-CFP cells were cultured, and at different time points, an aliquot of the conditioned medium was collected and assayed for Gluc activity. A linear increase of Gluc activity with respect to time was observed, showing the usefulness of this assay in monitoring cell proliferation (Fig. 1D). As Gluc is naturally secreted with $>95\%$ of the Gluc found in the conditioned medium (Fig. 1E),⁶ it offers two options for viability screening: (1) a single time point measurement at which the plated cells as well as the conditioned medium are assayed together, without the need to transfer an aliquot of the conditioned medium to new wells; or (2) time course measurements for functional analysis by assaying an aliquot of the conditioned medium from the same well at different time points, keeping the cells intact for confirmation analysis.

Optimizing the Gluc Assay for Functional Drug Screening

To optimize the Gluc cell-viability assay for HTS, several conditions were tested in a full 96-well plate format. Twenty microliters of aliquots of conditioned medium from Gli36-Gluc-CFP cells were aliquoted into 96-well plates. Initially, we observed high variation from well to well, in which the first well always gave the highest signal and the last well the lowest signal, being almost twofold lower than the first well. We attributed this variation to the instability and/or spontaneous auto-oxidation of the Gluc substrate coelenterazine. Upon incubation of coelenterazine (40 μ M) at different time points at

room temperature (RT) before using it, we observed that coelenterazine showed a very fast decay during the first 20–25 min and then became stable over the next hour (Fig. 2A). Therefore, coelenterazine was incubated for 30 min at RT before using it in all subsequent assays.

To reduce the cost of the Gluc assay for HTS, and as we had observed from a previous work that Gluc has a high turnover rate and so the signal of Gluc increases with increasing substrate concentration,⁵ we analyzed different concentrations of coelenterazine. We decided to proceed with 50 μ L of 1.2 μ M coelenterazine, which also gave a large signal over background ratio without significantly affecting the sensitivity of the Gluc assay (data not shown).

To determine the well-to-well and plate-to-plate variation for the Gluc cell-viability assay, Gli36-Gluc-CFP cells were plated in a 96-well plate. Twenty-four hours later, aliquots of the conditioned medium were transferred to a clean white 96-well plate and assayed for Gluc activity by injecting 1.2 μ M coelenterazine (final concentration; preincubated at RT for 30 min) and acquiring photon counts using a luminometer. We observed that the variability of the Gluc assay in three independent experiments was minimal with well-to-well and plate-to-plate variation being $<9\%$ (Fig. 2B and Table 2). This minimal variation includes pipetting error, coelenterazine instability, luminometer injection, and measurement errors.

We have also determined the robustness of the Gluc HTS assay by calculating the Z' factor as follows: Z' factor = $1 - [3(\text{SD}_{\text{PBS}} +$

Fig. 1. The Gluc bioluminescent assay. **(A)** An expression cassette for Gluc and CFP separated by an IRES element under control of CMV promoter was cloned into a lentivirus vector. **(B)** Transduction efficiency of Gli36 and U87 cells after infection with the vector in **A**. **(C, D)** Linearity of the Gluc assay with respect to cell number and time. Different amounts of Gli36-Gluc-CFP cells **(C)** or 1,000 cells **(D)** were plated in a 96-well plate and Gluc activity was assayed in 20 μ L aliquots of the conditioned medium at 24 h **(C)** or different time points **(D)**. **(E)** Intracellular versus conditioned medium levels of Gluc. Gli36-Gluc-CFP cells were plated in 96-well plates, and 24 h later, conditioned medium, viable cells, or both were assayed for Gluc activity. All experiments were performed in triplicates. Error bars represent standard deviation. Gluc, *Gaussia* luciferase; CFP, cyan fluorescent protein; IRES, internal ribosome entry site; CMV, cytomegalovirus.

$SD_{TRAIL}/(\mu_{PBS} - \mu_{TRAIL})$. A Z' factor of ≥ 0.5 indicates a robust assay.¹³ Gli36-Gluc-CFP cells, which are sensitive to TRAIL, were plated in a 96-well plate. Half of the plate received PBS, whereas the other half received 250 ng/mL TRAIL. Twenty-four hours later, 20 μ L aliquot of the conditioned medium from each well was transferred into a new 96-well plate and the Gluc activity was measured using 1.2 μ M coelenterazine. This experiment was repeated three times. We found that the Z' factor for the Gluc cell-viability assay ranged from 0.71 to 0.81, indicating a robust assay.

Gluc Assay for Monitoring TRAIL-Induced Apoptosis in GBM Cells

To determine whether the level of expression and secretion of Gluc into the conditioned medium can be used as a marker for cell death, we transduced Gli36 and U87 GBM cell lines with the lentivirus vector expressing Gluc and CFP. These cells were plated in a 96-well plate and treated with either PBS (control) or the human recombinant TRAIL (250 ng/mL). After 24 h, the Gluc bioluminescent activity was measured in the conditioned medium. TRAIL treatment resulted in $<25\%$ decrease in Gluc activity in the U87 cell line and $>60\%$ decrease in Gluc activity in Gli36 when compared with nontreated cells (Fig. 3A). This drop in Gluc expression also corresponded to the cytotoxicity observed using light and CFP fluorescent microscopy (data not shown). To correlate the Gluc viability measurements with a well-established assay, we used the Cell Titer-Glo assay, which is based on the quantification of the intracellular ATP level using re-

combinant firefly luciferase. Gli36 cells were treated with different concentrations of TRAIL and both assays were performed after 24 h on conditioned media (Gluc) or cell lysates (Cell Titer-Glo). There was no significant difference in cell viability measured by these two independent methods (Fig. 3B).

We then analyzed whether the Gluc reporter could be used as a functional assay to monitor cell death over time. Ten-thousand Gli36-Gluc-CFP cells were plated in a 96-well plate and treated with PBS or 250 ng/mL of recombinant TRAIL. The Gluc activity in the conditioned medium was monitored by assaying an aliquot of the conditioned medium at different time points. Again, the percentage decrease in Gluc activity in TRAIL-treated cells when compared with control cells decreased over time (up to 48 h), indicating that Gluc secretion can be used as a reporter to monitor cell viability and TRAIL-induced cell death in real time (Fig. 3C). Interestingly, at the 96 h time point, an increase in Gluc activity was observed in Gli36-Gluc-CFP cells, indicating an increase in cell number, which we attribute to the regrowth of a TRAIL-resistant subpopulation of the Gli36 cell line. In conclusion, the Gluc assay can be used to track the cytotoxic effect of TRAIL-induced apoptosis in GBM cells.

Small-Molecule Screening for Drugs That Sensitize GBM Cells to TRAIL

After having established a functional assay to monitor cell viability based on Gluc secretion, we screened for drugs that sensitize the resistant U87-Gluc-CFP cells to TRAIL-induced apoptosis. The

Fig. 2. Reproducibility of the Gluc assay. **(A)** Coelenterazine stability and auto-oxidation over time. Conditioned media from cells expressing Gluc were plated in a 96-well plate. Coelenterazine was mixed in phosphate-buffered saline (40 μ M) and used to assay triplicates of these wells at different time points. **(B)** Reproducibility of Gluc assay for functional screening. Gli36-Gluc-CFP cells were plated in 96-well plates, and after 24 h, Gluc activity was measured in 20 μ L of the conditioned medium using a luminometer after injecting 20 μ M coelenterazine. The experiment was performed in triplicate.

workflow of the entire screen of the 1,040 compound library (NINDS custom collection library) and hit validation is summarized in Figure 4A. U87-Gluc-CFP cells were plated in 96-well plates. In one set of plates, the cells were cultured in the presence of each drug at a final concentration of 1 μ M and 0.1% DMSO.³ In the second set of plates,

cells were incubated in the presence of both the drug (1 μ M) and TRAIL (50 ng/mL). This concentration of TRAIL gives ~20% cell kill on U87-Gluc-CFP cells. Aliquots of the conditioned medium were analyzed for Gluc activity at 48 h by adding coelenterazine and measuring photon counts using a luminometer. The drug hits were selected upon showing a >80% drop in photon counts when incubated with TRAIL, when compared with control cells cultured with 0.1% DMSO. The results from the primary screen are presented as a scatter plot in Figure 4B. Notably, at a concentration of 1 μ M, some of the drugs screened seemed to be toxic on U87 cells on their own (>80% decrease in normalized Gluc activity). Among those hits were two drugs already known to have anticancer activity, mitoxantrone¹⁵ and anisomycin,¹⁶ which confirms that Gluc is a robust tool for drug screening against tumor cells. In this primary screen, a total of 27 drugs were found to induce >80% decrease in Gluc activity in the presence of TRAIL, with <50% toxicity in the absence of TRAIL. These drugs were validated in a secondary screen in which 15 of these drugs were confirmed to sensitize U87 cells to TRAIL-induced cell death. The NINDS Custom Collection Library contained a total of

Table 2. Reproducibility of the *Gaussia* Luciferase Assay for Functional Screening

	Average (RLU)	SD (RLU)	Coefficient of Variation (%)
Plate 1	880,830	67,401	7.65
Plate 2	908,075	57,012	6.28
Plate 3	848,505	52,141	6.15
Interplate variation	875,659	43,811	9.8

SD, standard deviation.

Fig. 3. Gluc as a reporter to monitor TRAIL-induced cell death. **(A)** Gli36-Gluc-CFP and U87-Gluc-CFP were treated with 0 (black) or 250 ng/mL (gray) of TRAIL for 24 h. Gluc activity was measured in 20 μ L aliquots of the conditioned medium using a luminometer after adding 20 μ M coelenterazine. **(B)** Gli36-Gluc-CFP cells were treated with different concentrations of TRAIL. After 24 h, Gluc activity (black diamond) was measured in an aliquot of conditioned medium and cell lysates were assayed using the Cell Titer-Glo assay (gray rectangle). **(C)** Gli36-Gluc-CFP cells were treated with 250 ng/mL of TRAIL and the Gluc activity in the conditioned medium was monitored at different time points. The results are expressed as percentage of RLU in TRAIL-treated cells when compared with nontreated cells, the latter being 100%. All experiments were performed in triplicates. The error bars represent the standard deviation. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; RLU, relative light unit.

Fig. 4. Gluc functional screening assay. **(A)** A general scheme showing the different steps of the screening assay. **(B)** Scatter plot of the 1,040 compounds tested. Results are presented as percentage of Gluc expression in which the control (0.1% dimethyl sulfoxide) was set at 100%. **(C)** Statistical pie representing the different families of drug hits. Data are presented as percentage of drugs from the same family compared with the total drug hits. NINDS, National Institute of Neurological Disorders and Stroke. Color images available online at www.liebertonline.com/adt

seven drugs belonging to the family of cardiac glycosides. Interestingly, of the 15 hits that were confirmed in the secondary screen, 5 cardiac glycosides were revealed representing the largest family of drug hits obtained after the primary screen (Fig. 4C).

Validation of the Cardiac Glycoside Family for TRAIL Sensitization

The total set of seven cardiac glycosides was reanalyzed at a concentration of 0.25 μ M on U87-Gluc-CFP cells (Fig. 5A). At this concentration, the tested cardiac glycosides showed limited toxicity (<30%) on U87-Gluc-CFP cells on their own. However, when combined with TRAIL (50 ng/mL), >70% cell kill was observed for all cardiac glycosides tested, suggesting that a common chemical structure in the cardiac glycosides family is responsible for the observed TRAIL-sensitizing effect on brain tumor cells. For further confirmation, we used the Cell Titer-Glo assay on lysates from the same cells, which we initially collected from the supernatant for the Gluc assay. The RLU values obtained from the Cell Titer-Glo assay

were plotted against the Gluc RLUs, which showed a very tight correlation with $R^2 = 0.9769$ (Fig. 5B).

Next, four of these cardiac glycosides were randomly picked for further validation on primary GBM cells. GBM-Gluc-CFP cells were treated with different doses of lanatoside C, digitoxin, digoxin, and ouabain in the presence or absence of 50 ng/mL TRAIL (Fig. 5C). The Gluc data confirmed that these cardiac glycosides sensitize primary GBM cells to TRAIL-mediated cell kill. It is noteworthy that these four cardiac glycosides in the absence of TRAIL showed higher cytotoxicity on these primary cells when compared with the U87 cell line. Based on the Gluc values, we were able to calculate the IC_{50} of each drug defined as the concentration that yields a 50% decrease in Gluc expression in the presence of TRAIL when compared with the DMSO control (Table 3). Based on the IC_{50} calculation, lanatoside C was the least toxic on its own, whereas ouabain showed highest toxicity at 24 h after treatment. In the presence of TRAIL, the IC_{50} of lanatoside C, digoxin, ouabain, and digitoxin were 154, 70, 15, and 34 nM, respectively.

Fig. 5. Cardiac glycosides and TRAIL effect on GBM cells. **(A)** U87-Gluc-CFP cells were treated with 0.25 μ M of each cardiac glycoside in the presence (blue) or absence (brown) of 50 ng/mL TRAIL. Aliquots of the conditioned medium were assayed for Gluc activity after 24 h. The experiment was performed in triplicates. Error bars represent the standard deviation. **(B)** Correlation of the Gluc assay with Cell Titer-Glo. Cell lysates from **A** were analyzed using the Cell Titer-Glo assays. The % RLUs obtained using the Cell Titer-Glo was plotted against % RLUs obtained using the Gluc assay. **(C)** Analysis of different cardiac glycosides on primary GBM cells. GBM cells dissociated from 1 patient and expressing Gluc and CFP were treated with different doses of lanatoside C, digitoxin, digoxin, and ouabain in the presence or absence of 50 ng/mL TRAIL. Aliquots of the conditioned medium were assayed for Gluc activity at 24 h (no TRAIL, blue circles; TRAIL, red inverted triangle) and 48 h (no TRAIL, green diamonds; TRAIL, violet circles) posttreatment. The experiment was performed in triplicates and each data point is presented as percentage of Gluc expression when compared with untreated control, the latter being set at 100%. GBM, glioblastoma multiforme. Color images available online at www.liebertonline.com/adt

Table 3. IC₅₀ (nM) of All Cardiac Glycosides Tested in the Presence and Absence of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (50 ng/mL) as Determined by the *Gaussia* Luciferase Assay at 24 h Posttreatment

	No TRAIL	TRAIL
Lanatoside C	295	154
Digoxin	113	70
Ouabain	67	15
Digitoxin	96	34

IC₅₀, median inhibition concentration.

DISCUSSION

We have optimized the Gluc reporter gene assay for functional drug screening and showed it to be a useful tool for monitoring tumor cell viability. The Gluc substrate can be either dispensed directly on the treated cultured cells for a single time measurement, or aliquots of the conditioned medium can be analyzed at different time points to monitor cell proliferation or cell death in real time. The Gluc activity correlated directly with cell viability, as well as cell growth and proliferation. Compared with the firefly luciferase reporter gene assay, which is currently the gold standard assay for bioluminescence screening,^{17–20} Gluc offers several advantages. Gluc is naturally secreted, and therefore, it allows monitoring of cell viability by assaying aliquots of the conditioned medium over time, keeping the cells viable for validation purposes. This additive feature of the Gluc assay opens the door for screening of drug kinetics. In addition, firefly luciferase is not secreted, and therefore, an extra cell lysis step is required for efficient bioluminescent measurement, making time course experiments virtually impossible.²¹ This secreted luciferase can be easily used for noninvasive *ex vivo* monitoring of *in vivo* processes, including tumor growth and therapy, by measuring its activity in few microliters of blood.²² This allows a relatively easy testing and semithroughput validation of drugs *in vivo*.

Although the Gluc reporter assay could complement other cell viability assays and is suited for drug screening, it could not be used as a gold-standard measurement of cell death. The Gluc assay requires transduction of the reporter plasmid into cells, followed by gene expression, which is dependent on a transcription promoter, and therefore, its activity could be tempered by certain drugs. On the other hand, cell viability assays based on recombinant Fluc, which measures endogenous ATP levels, such as the Cell Titer-Glo (used in this study for conformational analysis), requires simple cell lysis without the need for reporter expression. Further, drugs that could induce endoplasmic reticulum stress or could impact the secretory pathway might interfere with Gluc secretion⁶ causing inaccurate bioluminescence read-outs. Hence, it is important to use alternative cell death assays for validation analysis. Unlike Fluc, which catalyzes a glow-type bioluminescence, Gluc catalyzes a flash-type reaction, and therefore, a luminometer with

a built-in injector must be used for screening. This problem could be overcome by using reagents (e.g., GAR-2; Targeting Systems, El Cajon, CA) or mutant variants of Gluc that stabilize the light output in the presence of detergents such as Triton X-100.²³

The noninvasive nature of the Gluc assay for drug screening is also suited for multiplexing with other genomic or proteomic assays. Further, this approach can be used in gene-targeted drug screening with reporter vectors containing DNA-binding sequences of the gene of interest driving the expression of Gluc. For instance, the transcription factors p53 and nuclear factor- κ B (NF κ B) are important targets for cancer drug development. A p53 reporter vector was used to identify small molecules affecting the transcriptional activation of this gene.¹⁷ Recently, we developed an NF κ B reporter system amenable for drug screening, which can be used in combination with the optimized Gluc drug screening assay developed here to identify NF κ B inhibitors.²⁴ In conclusion, the Gluc assay can help expedite drug identification and development and, subsequently, functional assessment of new treatment regimens in animal models.

Using the Gluc assay, we identified the family of cardiac glycosides as sensitizers for GBM cells to TRAIL-induced cell death. This family of compounds on their own has been suggested as potential therapeutics against cancer.^{25,26} However, these drugs have not reached the clinic because of the requirement of a relatively high dose for activity, leading to cardiotoxicity. Here, we showed that a low dose of these cardiac glycosides in combination with a low dose of TRAIL serves as an efficient therapy against GBM cells. As both cardiac glycosides as well as TRAIL have been approved by the Food and Drug Administration for use in humans, the combination of this therapy can be easily translated to clinical use.

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DISCLOSURE STATEMENT

None of the authors of this work has any conflicting interests.

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