

APPLICATION NOTE

Assessing viability and real-time apoptosis/necrosis in an *in vitro* model of malignant glioma

Silvia Vidali, PhD | Application Scientist | Molecular Devices

Emanuele Giordano | Application Scientist | Molecular Devices

Introduction

Malignant gliomas, a subset of aggressive brain tumors, represent a formidable challenge in the realm of oncology due to their invasive nature and resistance to conventional treatments. As researchers strive to unravel the intricacies of these tumors, assessing the viability and understanding the real-time dynamics of apoptosis and necrosis in malignant glioma cells emerges as a crucial focal point. The ability to discern the survival status of these cells and monitor their programmed cell death in real time is instrumental in developing targeted therapeutic strategies.

In this context, advancements in technology have paved the way for innovative approaches to assess viability, apoptosis, and necrosis, providing researchers with a more comprehensive understanding of the cellular responses within malignant glioma. This exploration into the dynamics of cell survival and programmed cell death is not only pivotal for fundamental research but also holds significant implications for the development of effective treatment modalities that can specifically target the unique characteristics of malignant gliomas.

In this concise overview, we outline the fundamental steps involved in a miniature-scale compound screening tailored for cancer and pharmacological research. The initial screening phase encompassed the application of diverse compounds at varying concentrations and time points, followed by performance of the CellTiter-Glo® 2.0 Cell

Benefits

- Highly sensitive luminescent readout enables rapid assessment of viability for larger compounds screening
- Easy real-time monitoring of apoptosis and necrosis simplifies the understanding of compounds toxicity behavior
- The viability assay, coupled with subsequent determination of necrosis and apoptosis, yields a comprehensive understanding of how selected compounds impact malignant gliomas

Viability Assay (Promega). This preliminary step enables researchers to identify the most efficacious compound and determine the optimal concentration for subsequent in-depth and sophisticated studies.

We subsequently delved further into the assessment of viability, extending the investigation to the impact of the selected compound(s) on apoptosis and necrosis. To achieve this, we used the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega). Specifically, our study focused on U-87 MG malignant glioma cells, and the experimental data were acquired using the SpectraMax i3x Multi-Mode Microplate Reader.

By employing this systematic approach, we aimed to provide insights into the compound screening process and showcase the applicability of advanced assays, thereby facilitating nuanced investigations into the efficacy and mechanisms of potential therapeutic agents in the context of cancer research.

Assays used

CellTiter-Glo® 2.0 Cell Viability Assay

This assay is a homogeneous, bioluminescent assay for determining the relative number of viable cells in culture. The method is based on quantitation of the ATP present in the well, an indicator of metabolically active cells (Figure 1). A volume of CellTiter-Glo 2.0 reagent equal to the volume of culture media is added to each well, and after a very brief incubation, luminescence is measured on a microplate reader.

RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay

Promega has designed an assay to simultaneously measure apoptosis and necrosis in real-time. The binding of two annexin V fusion proteins containing complementary subunits of NanoBiT® luciferase to exposed phosphatidylserine on the outer leaflet of cell membranes during the apoptotic process enables the generation of a luminescent signal. Upon loss of membrane integrity, a DNA-binding dye enters the cell and generates a fluorescent signal (Ex 485 nm / Em 525–530 nm) that indicates necrosis (Figure 2).

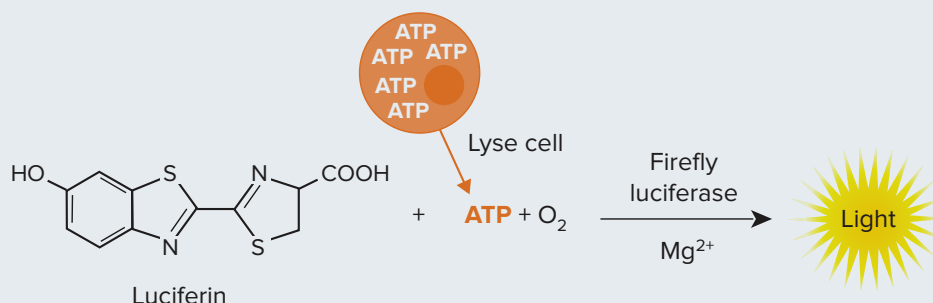


Figure 1. Schematic of the ATP-based luciferin assay mechanism.

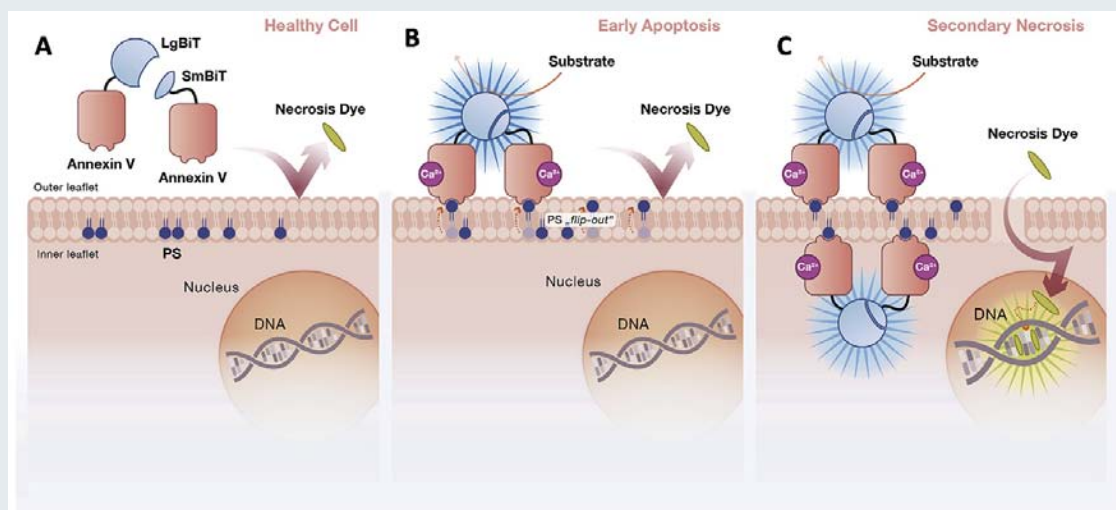


Figure 2. In healthy cells the Annexin V-LgBiT and Annexin V-SmBiT (NanoBiT®) fusion proteins are too far apart, and luminescence is negative. The cell-impermeant pro-fluorescent DNA dye (Necrosis Dye) does not bind to DNA and the fluorescence signal is also negative (A). In early apoptosis, luminescence remains low until PS exposure brings the Annexin V fusion proteins close together, reconstituting a functional luciferase, while the Necrosis Dye is excluded from cells. Luminescence is positive and fluorescence is negative (B). During secondary necrosis, loss of membrane integrity allows for binding of the Necrosis Dye to DNA, yielding a fluorescent signal. Here, both luminescence and fluorescence are positive (C).

Materials

- U-87 MG human malignant glioma cell line from ATCC (HTB-14)
- Growth medium: ATCC-formulated Eagle’s Minimum Essential Medium (ATTC cat. #30-2003), fetal bovine serum to a final concentration of 10% (Gibco cat. #11573397) and penicillin-streptomycin 100x (Gibco Penicillin-Streptomycin 10,000 U/mL, cat. #15140-122).
- 96-well Black/Clear Bottom Microplate (Corning cat. #3603)
- CellTiter-Glo® 2.0 Assay (Promega cat. #G9241)
- RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay (Promega cat. #JA1011)
- Antimycin A from Streptomyces sp. (Merck cat. #A8674).
- Staurosporine solution, from Streptomyces sp. (Merck cat. # S6941)
- Doxorubicin hydrochloride (Merck cat. #4229251)
- Taxol (paclitaxel) (Merck cat. # PHL89806)
- SpectraMax i3x Multi-Mode Microplate Reader. Equipped with the Luminescence detection cartridge (LUM96) (Molecular Devices cat. #0200-7014).

Methods

Compound treatment

10,000 U-87 MG cells per well were seeded (100 µL) in a black-wall, clear-bottom microplate and allowed to attach for 24 hours. Quadruplicate wells of cells were subjected to serial dilutions of antimycin A, staurosporine (ranging from 3 µM to 0.03 µM), doxorubicin, Taxol (ranging from 100 µM to 1 µM), or the corresponding vehicle for each compound, namely 0.1% ethanol for antimycin A and 0.3%

dimethyl sulfoxide (DMSO) for the other compounds. For the viability assay, media was removed and compounds in media were added at their final concentration with a total volume of 100 µL, and cells were then incubated for 24 or 72 hours. Following the incubation period, cells were processed for the CellTiter-Glo® 2.0 Assay.

For the apoptosis/necrosis assay, media was removed from the wells and 100 µL of compounds in media were added at 2x concentration. Immediately after the addition of the compounds, the Detection Reagent was added in a 1:1 ratio. The strong red color of doxorubicin prompted the use of lower doses (ranging from 1 µM to 0.001 µM) for the apoptosis/necrosis assay, as it was covering the luminescent signal.

Viability assay

After the specified incubation at 37°C with 5% CO₂ for 24 or 72 hours, the CellTiter-Glo® 2.0 assay was conducted. The assay reagent was added at a 1:1 ratio to each well (100 µL + 100 µL). The plate was inserted into the SpectraMax i3x reader and underwent high-speed, double orbital shaking for 2 minutes to induce cell lysis. Subsequently, the plate was incubated at room temperature for 10 minutes to stabilize the luminescent signal before measurement.

Apoptosis/necrosis assay

The RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay was employed to discern the nature of the cytotoxic effects induced by the compounds. Assay reagents were added immediately after compound treatment, and the plate was shaken for 30 seconds (high-speed, double orbital) in the SpectraMax i3x reader prior to reading. Measurements in luminescence mode for apoptosis, and fluorescence mode for necrosis, were taken at T0, followed by subsequent readings at 1, 3, 6, and 24 hours, with intervals of incubation in the tissue culture incubator.

Plate reader settings	Luminescence	Fluorescence
Wavelength	Lumi 96 cartridge	Ex 485 nm, 9 nm bandwidth Em 535 nm, 15 nm bandwidth
Plate Type	96 Well Costar blk/clrbtm	96 Well Costar blk/clrbtm
PMT and Optics	Detection: Off-Stop and Go	PMT gain: [DefaultP]
Integration time	1000 ms	6 flashes/read
Read from Bottom	No	Yes

Results

Viability assessment

An initial compound screening was conducted to evaluate the potential toxic effects on cells using a viability assay. The CellTiter-Glo® 2.0 assay, a luminescence test that detects the cellular viability marker ATP by utilizing the ATP-dependent firefly luciferase reaction, served as the measurement tool. The luminescent signal produced is directly proportional to the number of viable cells.

Following treatment with antimycin A, there was no significant decrease in viability after 24 hours. However, a discernible reduction in luminescent signal was observed after 72 hours compared to untreated cells (CTRL) and vehicle-treated cells (EtOH), with no noticeable variation among the tested compound concentrations (Figure 3).

In contrast, cells treated with staurosporine and doxorubicin exhibited a dose-dependent reduction in signal as early as 24 hours, compared to untreated and DMSO-treated cells. This reduction became more pronounced at 72 hours, reaching very low signal levels at the highest concentrations of staurosporine (Figure 3).

Conversely, Taxol treatment induced a slight decrease in signal at 24 hours and a more substantial decrease at 72 hours. However, no significant dose dependency was evident.

All four compounds under scrutiny demonstrated the potential to impede cell growth and viability. Consequently, a single concentration of each compound was chosen for further investigation in order to explore its cytotoxic effects on cells.

RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay

An increase in apoptosis was observed in both untreated and treated cells across all conditions. Control and vehicle accounted to about 1000 relative luminescence units (RLU) at 24 hours, indicating the baseline apoptosis rate of these cells in this culture conditions. Similar results were observed for the secondary necrosis rate (Figure 4 A–H).

Cells treated with antimycin A exhibited comparable apoptosis levels to control and EtOH-treated cells, suggesting that antimycin A does not activate the apoptosis pathway (Figure 4 A). A slight, but not significant, increase in secondary necrosis was observed at 24 hours in treated cells, compared to the vehicle (Figure 4B). Conversely, a surge in apoptotic luminescent signals, compared to the control and vehicle, was evident as early as 3 hours in cells treated with staurosporine (our positive control for apoptosis) and increased even further after 6 hours. By 24 hours apoptotic signal did not significantly increase further (Figure 4C). Nevertheless, secondary necrosis was significantly increase at 24 hours compared to the vehicle. These data confirm that staurosporine is a strong inducer of apoptosis at the early time points (Figure 4D).

Doxorubicin treatment increased apoptosis by 24 hours, compared to vehicle, but not at earlier timepoints, exhibiting a delay in activation of apoptosis compared to staurosporine (Figure 4E). Even at low concentrations, doxorubicin's dark red color apparently had a quenching effect on the fluorescence signal used to detect necrosis (Figure 4F). In drug screening, an operator must take into account the potential effect of strongly colored candidate compounds on the assay readout.

Finally, Taxol induced a significant increase in apoptotic signal after 6 hours, which continued to 24 hours (Figure 4G). Interestingly, secondary necrosis was not affected (Figure 4H).

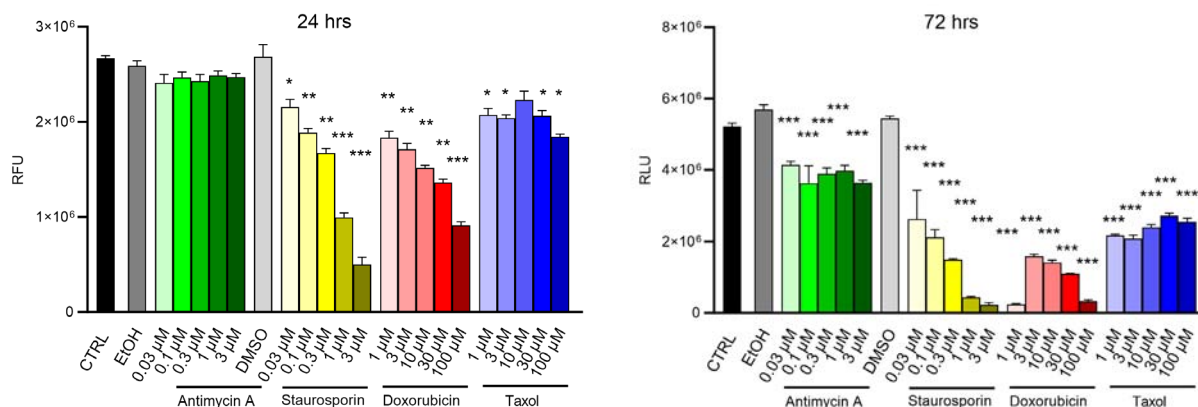


Figure 3. Graphs show the relative luminescence unit (RLU) of cells treated with serial dilutions of Antimycin A (3 μM–0.03 μM), Staurosporine (3 μM–0.03 μM), Doxorubicin (100 μM–1 μM) and Taxol (100 μM–1 μM) for 24 hours or 72 hours. Cell viability was assessed using the CellTiter-Glo® 2.0 assay. Mean ± SD, n = 4 or 5 wells. Brown-Forsythe and Welch ANOVA test. Significance is given for treated vs. corresponding vehicle. * p < 0.05; ** p < 0.01; *** p < 0.001. CTRL, Control; DMSO, dimethyl sulfoxide; EtOH, ethanol.

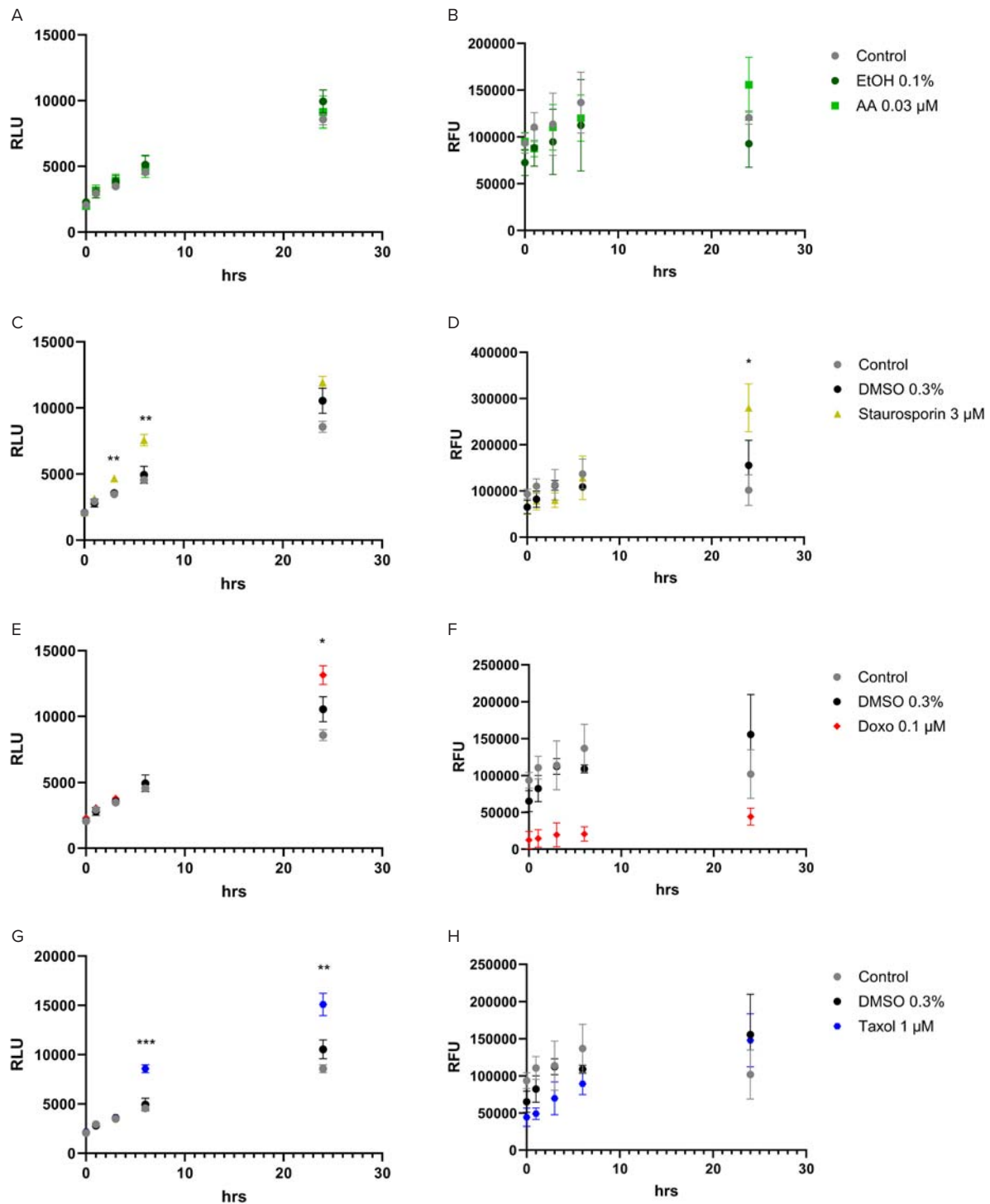


Figure 4. Graphs show the relative luminescence unit (RLU) (A, C, E, G) and the relative fluorescence unit (RFU) (B, D, F, H) of cells treated with media only (control) (A–H), vehicle EtOH (A, B) or DMSO (C–H), Antimycin A 0.03 μM (A, B), Staurosporine 3 μM (C, D), Doxorubicin 0.1 μM (E, F) or Taxol 1 μM (G, H). Cells were then processed for the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay, and measurement were executed at time 0, 1, 3, 6 and 24 hours. Mean ± SD, n=3–4 wells. Two-way ANOVA test. Significance is given for treated vs. corresponding vehicle. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. CTRL, Control; DMSO, dimethyl sulfoxide; EtOH, ethanol.

Conclusions

In this application note, we showcase the utility of the CellTiter-Glo® 2.0 Cell Viability Assay paired with the SpectraMax i3x reader, offering a powerful solution for the preliminary screening of compounds. The use of a relatively simple cell viability assay enables one to identify candidate compounds at concentrations that merit further investigation.

In cancer research, the subsequent phase of investigation often involves discerning whether diminished viability stems from cell cycle arrest, induction of apoptosis, or necrosis. Delving deeper with the RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay provides additional information that elucidates the type of cell death induced by selected compounds. Armed with these insights, researchers can strategically choose additional molecular analyses to precisely elucidate the mechanisms of action of the identified compound(s).

The compact design of the SpectraMax i3x reader provides an all-encompassing solution for investigating both fluorescence and luminescence within the same instrument. Its built-in shaking capability negates the need for external shakers for the assays featured here. Reader control is handled through SoftMax Pro software. Industries governed by GxP regulations can place their trust in the SoftMax Pro GxP software, ensuring seamless compliance with GxP requirements.

Contact Us

Phone: +1.800.635.5577
Web: www.moleculardevices.com
Email: info@moldev.com
Check our website for a current listing of worldwide distributors.

Regional Offices

USA and Canada	+1.800.635.5577	Taiwan/Hong Kong	+886.2.2656.7585
United Kingdom	+44.118.944.8000	Japan	+81.3.6362.9109
Europe*	00800.665.32860	South Korea	+82.2.3471.9531
China	+86.4008203586	India	+91.73.8661.1198

**Austria, Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain, Sweden, Switzerland and United Kingdom*