Comparison of Oridonin Cytotoxicity in U-2 OS and HepG2 Cells

Using the BioSpa™ 8 to Manage Repeated Reagent Additions to Live Cells

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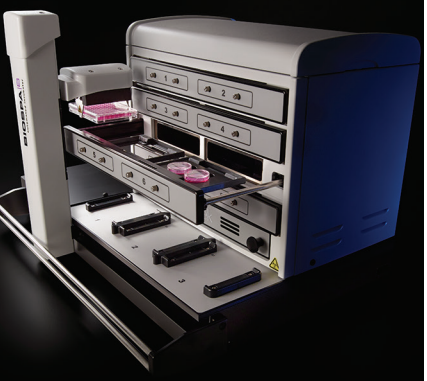

Key Words:

Automation
Live Cell Assays
Cytotoxicity
Oridonin

The timed exposure of live cells to compound is a long standing experimental protocol for drug discovery. The speed and concentration at which compounds elicit a response to cells in vitro provide important clues as to their efficacy in vivo. Here we describe the use of a BioSpa™ 8 Automated Incubator to interface a MultiFlo™ FX Multi-Mode Dispenser to a Cytation™ 5 Cell Imaging Multi-Mode Reader to perform automated cytotoxicity testing.

Introduction

Toxicity is one of the leading causes of attrition in small molecule drug discovery, so testing putative drugs using cell-based cytotoxicity assays is an important part of pre-clinical R&D. The timed exposure of live cells to compound titrations is often used as the benchmark for cytotoxicity. While originally quantitative absorbance or fluorescence based cytotoxicity assays were employed, high content analysis using microscopy is becoming more popular due to the wealth of information garnished from this approach. High content assays have further progressed such that many of these assays are performed with live cells rather than fixed and antibody stained specimens. Live cells can provide critical temporal information that static fixed cell staining techniques cannot.

Imaging cells in microplates to assess the effect of compound exposure over time can be approached in two different ways. Cells can be treated with drug compounds initially and repeatedly imaged at a known interval, or, compounds can be added to cells at successive time points and the plate imaged one time. With repeated imaging true kinetic results can be obtained, as the effect of the compound is observed on the same cells with each data time point.

Figure 1. **Chinese herb Isodons rubescens indigenous to south and central China.** Photo supplied by. I, KENPEI, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=2912263.

However, repeated exposure of cells to high intensity light for imaging can affect cell viability independent of any compound effect. This is particularly true if a nuclear stain such as Hoechst 33342 is used, as the near UV light used for excitation is more energetic than visible light. Adding compound at different intervals using separate wells for different exposure times avoids the cytotoxicity associated with repeated light exposure. This method requires repeated reagent additions and because the wells of each plate are only imaged once, more wells are required in order to provide statistical confidence to the data. Due to the manual intervention required this experimental setup is often avoided. However, with an affordable automated system, the multiple plates can be run concurrently without the need of manual intervention.

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To demonstrate the value of this system, we have evaluated the effects of Oridonin on U-2 OS and HepG2 cells using the reagent CellTox™Green. Oridonin, a natural tetracycline diterpenoid isolated from Chinese herb Isodons rubescens, (Figure 1) has been reported to be a potent cytotoxic agent against a wide variety of tumors. Oridonin has been shown to induce potent growth inhibition on human breast cancer cells MCF-7 and MDA-MB-231 in a time- and dose-dependent manner though cell cycle arrest at the G2/M phase [1]. Here we describe the use of the BioSpa™ 8 to manage several plates such that reagents are added periodically and the plates imaged such that both reagent concentration and exposure time are varied. In addition to plate transfer and scheduling the BioSpa 8 also serves to provide environmental control in between process steps.

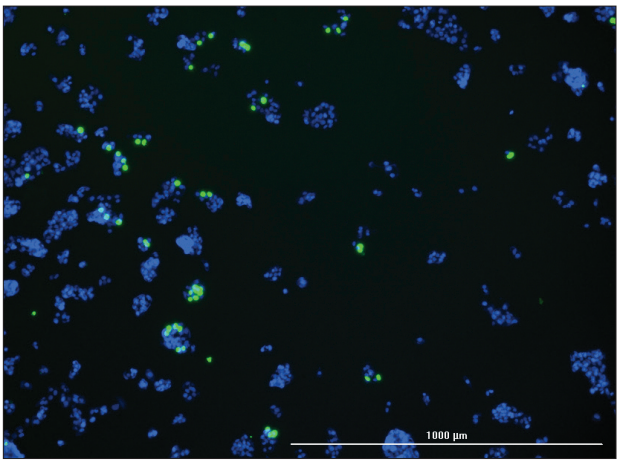

Figure 2. **Representative 4X image of HepG2 cells stained with Hoechst 33342 and CellTox™ Green.** Cells were treated with 30 µM oridonin for 6 hours, then stained with Hoechst 33342 (1 µg/ml) and CellTox™ green for 30 minutes. Digital microscopic images (4x) were made using Cytation 5.

Materials and Methods

Cell Culture

U-2 OS and HepG2 cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black sided clear bottom 96-well microplates at 5,000 cells per well.

Imaging

Cultures were imaged using a Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) Configured with DAPI, and GFP cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cubes uses a 377/50 excitation filter and a 447/60 emission filter, while the GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter.

Image Analysis

Two-color digital images were overlaid using Gen5 software. Digital preprocessing was used to subtract background fluorescence from each image prior to analysis. After background subtraction, the Hoechst 33342 nuclear stain signal was used to establish a mask identifying individual cells which could then be counted by Gen5 image analysis software. A blue fluorescence threshold of 2200 was used to define the mask. This same mask was then used in the green channel to assess cytotoxic cells using CellTox Green™ fluorescence. Cells that emitting green fluorescence above a threshold of 10,000 fluorescence units where considered cytotoxic cells and counted as such. (Figure 2).

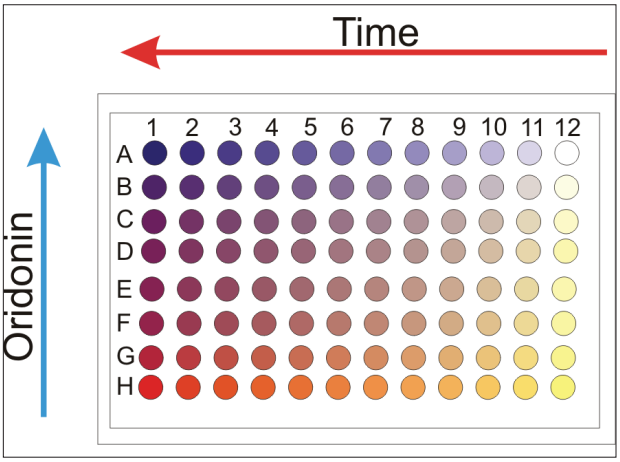

Figure 3. **Plate map configuration of Process Centric Oridonin cytotoxicity experiments.** Oridonin is added to the plate using the MultifFlo FX peripump such that different concentrations of drug are added with each of the eight separate dispense tubes in rows A-H. Drug is added at different times with each strip 1-12. Well A1 would have the highest drug concentration and longest exposure time, while well H12 would have the lowest drug concentration and the least amount of exposure time.

Experimental Design

Oridonin is added to the plate using the MultiFlo™ FX peripump. Different concentrations of drug are added with each of the eight separate dispense tubes in rows A-H; row A having the highest oridonin concentration, while row H is not treated with oridonin (Figure 3). also, compound additions are made at different times for each strip 1-12 (Figure 4). All plates are then imaged once, 24 hours after the initial addition of oridonin dosage. In this scheme, well A1 would have the highest drug concentration and drug exposure time, while well H12 would have the lowest drug concentration and the least amount of drug exposure time (Figure 3).

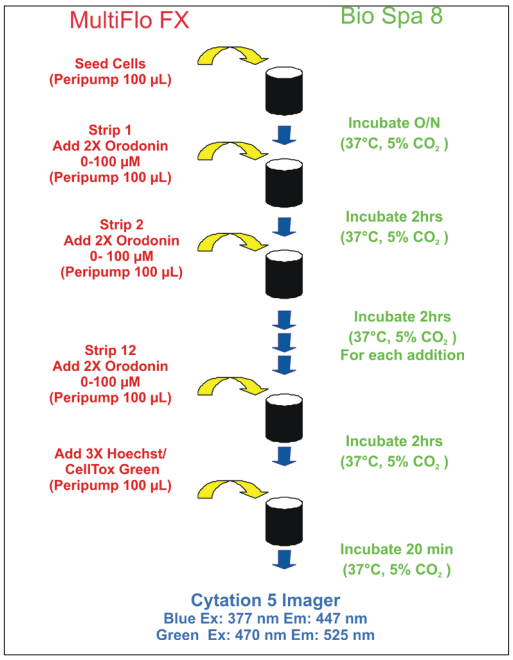

Figure 4. **Assay Process steps of a Cytotoxicity Assay with Multiple Reagent Additions.** A series of strip dispense routines were carried out with the MultiFlo FX to add various concentrations (0-100 µM) of Oridonin every 2 hours to U-2 OS or HepG2 cells in 4 separate plates. Plates are incubated in the BioSpa 8 at 37 °C, with a humidified 5% CO_2 atmosphere between reagent additions. After 24 hours Hoechst 3342 and CellTox™ Green stains are added and the plates imaged in the DAPI and GFP channels with a Cytation 5 Cell Imager multi-mode reader.

The assay process is programmed in BioSpa 8 software, which schedules routines from LHC software for the MultiFlo FX steps and Gen5 software for Cytation 5 imaging steps. This process schedule is displayed in a Gantt chart designating the start and finish time for each plate which is generated before the run begins (Figure 5). Estimated times for each process step are also determined and displayed. During the actual run a tracer is shown that indicates the current point in the run the system is at, as well as tracer lines for temperature, CO_2 and humidity.

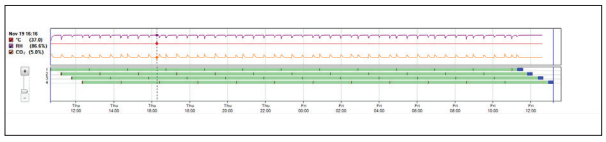

Figure 5. **Gantt chart of a BioSpa 8 cytotoxicity assay session with multiple reagent dispenses.** A series of strip dispense routines are carried out with the MultiFlo FX to add various concentrations (0-100 µM) of oridonin every 2 hours to U-2 OS and HepG2 cells in 4 separate plates. Plates are incubated in the BioSpa 8 at 37 °C, with a humidified 5% CO_2 atmosphere between reagent additions. After 24 hours, the plates are stained and imaged. Gas levels and humidity monitor traces are also present.

Results

Higher dosages of oridonin demonstrated measureable cytotoxicity beginning at approximately 16 hrs after the initiation of exposure, while low concentrations do not exhibit an increase in cytotoxicity until 20 hours (Figure 6).

Likewise, with increasing exposure times, more cytotoxicity is observed regardless of the drug concentration. This can be observed when drug titrations are grouped based on exposure time (Figure 7).

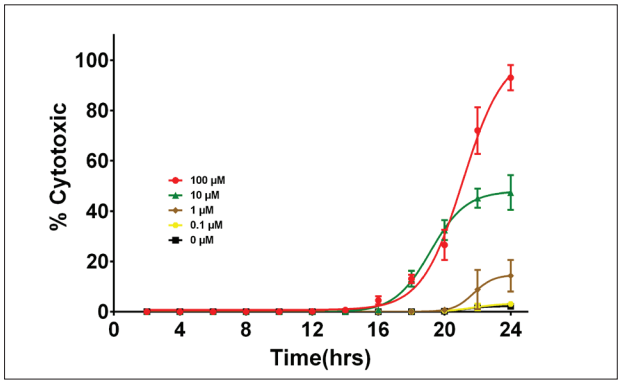

Figure 6. **Effect of Exposure time of Oridonin on Cytotoxicity.** U-2 OS cells were treated with various concentrations of oridonin and assayed for cytotoxicity after different exposure times. Results are expressed as a percent of the total number of cells counted from imaged based object counting of Hoechst 33342 stained nuclei. Data points reflect the mean of 4 determinations from different plates.

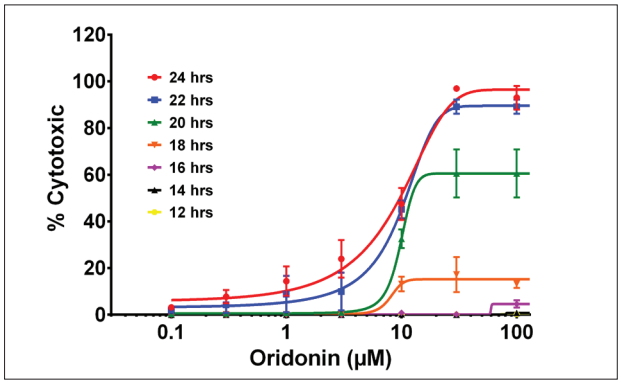

Figure 7. **Effect of Oridonin Concentration on Cytotoxicity.** U-2 OS cells were treated with various concentrations of oridonin and assayed for cytotoxicity after different exposure times. Results are expressed as a percent of the total number of cells counted from imaged based object counting of Hoechst 33342 stained nuclei. Data points reflect the mean of 4 determinations from different plates.

Oridonin also results in a loss of cell number in U-2 OS cells over a 24 hour period of time. The short time interval relative to the cell doubling time suggests that a true loss of cells through lysis is occurring rather than an inhibition of cell growth. Approximately 50% of the U-2 OS cells are lost by 24 hours with the oridonin concentrations tested (Figure 8).

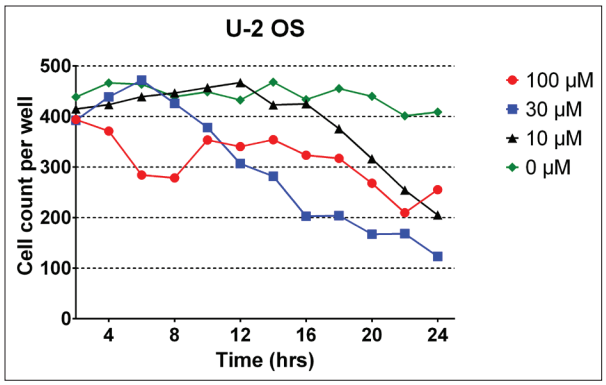

Figure 8. **U-2 OS Cell counts with Oridonin treatment.** Cell number was determined by object counting Hoechst 33342 stained U-2 OS cell nuclei. Each data point represents the mean of 3 data points each from a separate microplate.

HepG2 cells appear to be more resistant to oridonin than U-2 OS cells. While very high concentrations of oridonin (30 and 100 µM) caused a significant loss of cells, with a lower dose (10 µM) of oridonin little change in the number of HepG2 cells was observed (Figure 9).

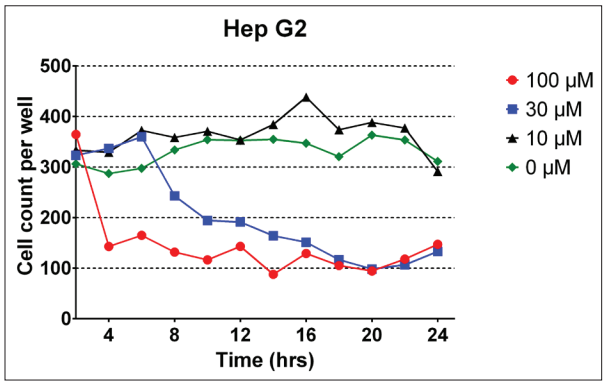

Figure 9. **HepG2 Cell counts with Oridonin treatment.** Cell number was determined by object counting Hoechst 33342 stained HepG2 cell nuclei. Each data point represents the mean of 3 data points each from a separate microplate.

Comparing the cytotoxicity of 10 µM oridonin in U-2 OS and HepG2 cells over time, significant cytotoxicity is observed in U-2 OS cells beginning at approximately 14 hours of exposure. In HepG2 cells very little cytotoxicity can be seen even with 24 hours of compound exposure (Figure 10).

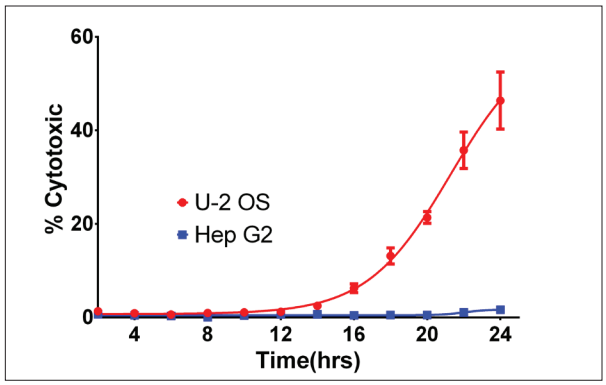

Figure 10. **Comparison of Oridonin Cytotoxicity on U-2 OS and HepG2 cells.** U-2 OS and Hep G2 cells were treated with 10 µM oridonin and assayed for cytotoxicity after different exposure times. Results are expressed as a percent of the total number of cells counted from imaged based object counting of Hoechst 33342 stained nuclei. Positive cell nuclei exhibit a mean green fluorescence greater than 10,000. Each data point represents the mean of 3 data points each from a separate microplate.

Oridonin compound titrations with a 24 hour exposure corroborate these findings. Substantial cytotoxicity is observed with U-2 OS cells at much lower compound concentrations than seen with HepG2 cells (Figure 11). The percentage of cytotoxic U-2 OS cells is twice that seen with HepG2 cells. Interestingly, at 10 µM oridonin concentration the fold difference between the two cell lines is almost 40 fold, which suggests a possible anti port system providing some of the drug resistance with HepG2 cells. Once the transport system is fully engaged, the cytotoxic effects of oridonin become manifest in HepG2 cells. The significant loss of cells at 100 µM oridonin for both cell lines also agrees with this.

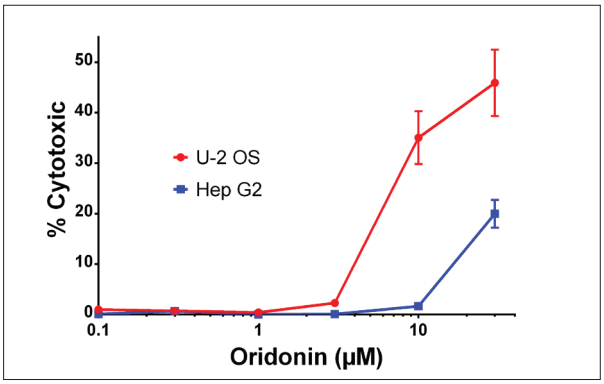

Figure 11. **Effect of Oridonin Concentration on Cytotoxicity.** U-2 OS and Hep G2 cells were treated with various concentrations of oridonin and assayed for cytotoxicity after a 24 hour exposure. Results are expressed as a percent of the total number of cells counted from imaged based object counting of Hoechst 33342 stained nuclei. Positive cell nuclei exhibit a mean green fluorescence greater than 10,000. Each data point represents the mean of 3 data points each from a separate microplate.

Discussion

These data indicate that the BioSpa 8 robotic incubator is capable of performing unattended live cell experiments across an entire 24-hour period. Several plates were moved to the MultiFlo™ FX for reagent addition and back to the incubator at 2-hour intervals. This resulted in the exposure of live cells to oridonin for different time intervals on the same microplate. At the end of the reagent exposure, stains to identify total cells, as well as cells exhibiting cytotoxicity were added and the plates digitally imaged with a 4x objective.

Differences in cytotoxicity observed between different cell lines are not an unexpected finding. While both HepG2 and U-2 OS cells are human derived cell lines, they are from completely different tissues. HepG2 cells are derived from a hepatic carcinoma [3], while U-2 OS cells were originally derived from bone osteosarcoma [4]. These experiments demonstrated that HepG2 cells were more resistant to the cytotoxic effects of oridonin than U-2 OS cells.

Compound dose and exposure studies are a necessary component of the drug discovery process. Small scale experiments, which are typically performed manually, require continual intervention throughout the entire experiment, which with live cell assays can run 24-48 hours. Normal procedure for these experiments is to limit the number of data points during the night time hours in order to avoid overnight stays in the laboratory. With the use of the BioSpa™ 8 Automated Incubator the experimental design regarding time points need not be directly linked to normal daytime working hours. Instead, unattended operation through the entire experiment is possible.

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