A Flow Cytometric Clonogenic Assay Reveals the Single-Cell Potency of Doxorubicin

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ABSTRACT: Standard cell proliferation assays use bulk media drug concentration to ascertain the potency of chemotherapeutic drugs; however, the relevant quantity is clearly the amount of drug actually taken up by the cell. To address this discrepancy, we have developed a flow cytometric clonogenic assay to correlate the amount of drug in a single cell with the cell's ability to proliferate using a cell tracing dye and doxorubicin, a naturally fluorescent chemotherapeutic drug. By varying doxorubicin concentration in the media, length of treatment time, and treatment with verapamil, an efflux pump inhibitor, we introduced 10^5–10^{10} doxorubicin molecules per cell; then used a dye-dilution assay to simultaneously assess the number of cell divisions. We find that a cell's ability to proliferate is a surprisingly conserved function of the number of intracellular doxorubicin molecules, resulting in single-cell IC_{50} values of 4–12 million intracellular doxorubicin molecules. The developed assay is a straightforward method for understanding a drug's single-cell potency and can be used for any fluorescent or fluorescently labeled drug, including nanoparticles or antibody–drug conjugates. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:4409–4416, 2015

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INTRODUCTION

Routinely used *in vitro* proliferation assays provide a high-throughput method for evaluating the potency of chemotherapeutic drugs.[1,2] Typical proliferation assays use the bulk media concentration to determine the drug potency (i.e., IC_{50} or IC_{90}). However, the drug concentration on a media volumetric basis would need to be freely in equilibrium with the drug's intracellular target for this to truly represent the drug's intrinsic potency. This is not true in almost any case as drugs encounter membranous diffusion barriers and may be substrates for active uptake or efflux transporters.[3] The amount of drug internalized into the cell is a more physiologically relevant basis for comparison than the bulk media concentration[4,5] especially when considering drug delivery systems that involve endosomal transport and processing steps, such as antibody–drug conjugates (ADCs) or liposome drug delivery systems.[6] It is now within the purview and capability of the drug designer to attempt to alter a drug's interaction with these transport and processing machineries, in order to attain more efficient delivery on target. However, a key piece of information in such cases is the number of drug molecules on target necessary for the desired effect (e.g., how many doxorubicin molecules does it take to kill a cell?). This information is not directly available from potencies determined on a media-volume basis. The assay described herein uses the amount of drug in an individual cell as the basis for cellular response rather than the drug concentration in the cell growth media.

Standard chemotherapy potency assays include non-clonogenic assays that are based on changes in cell membrane permeability (Lactate Dehydrogenase or Trypan Blue), mitochondrial function (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or WST-1 Assay), or markers for early (Annexin V) or late apoptosis (Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or cytochrome *c*).[7] In contrast, clonogenic assays measure a cell's ability to proliferate after treatment. Traditionally, proliferation is measured by counting clones that have grown out after cells have been plated at low density.[8] Clonogenic assays capture all types of cell death and include cell growth after reversible damage, whereas non-clonogenic assays measure acute cellular toxicity, often specific to one type of cell death. As clonogenic assays capture the integrated effect of many different types of cellular response to drug treatment, we focused on this assay type.

Here, we develop a flow cytometric dye-dilution clonogenic assay to determine the relationship between the amount of drug in a single cell and the cell's ability to proliferate. Flow cytometry enables high-throughput screening of thousands of individual cells, resulting in analysis on a single-cell level rather than a bulk population level. The assay uses a cell tracing dye and a fluorescent drug. A cell tracing dye is used to track cell proliferation via dye dilution. All cells are initially stained with

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dye and the dye is diluted in half with each cell division. A fluorescent drug is used in order to measure the amount of drug taken up by each cell.

In this work, we used doxorubicin, a standard chemotherapeutic drug,[9] which is also naturally fluorescent,[10] as a model drug to demonstrate application of the assay. Doxorobucin is known to bind DNA and inhibit topoisomerase II[9] and is widely used as a front-line therapy for a number of different types of cancer.[11]

METHODS

Cell Lines and Materials

Eight different cell lines were used in this work: BT-474, HCT-15, HT-29, IGROV-1,[12] MDA-MB-231, NCI-N87, SK-BR-3, and T-47D. Cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia). All cell lines except HT-29 and SK-BR-3 were grown in RPMI (Corning Mediatech, Manassas, Virginia) supplemented with 10% fetal bovine serum and 5% penicillin–streptomycin. HT-29 and SK-BR-3 cells were grown in Dulbecco's Modified Eagle Medium (Corning Mediatech) and McCoy's 5A Medium Modified (Lonza, Basel, Switzerland), respectively, supplemented in the same way. Doxorubicin hydrochloride and verapamil were purchased from Sigma (St. Louis, Missouri).

Assay Set-Up

Cells were stained using CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific (Invitrogen), Grand Island, New York) following the "Standard Method for Labeling Cells in Suspension" as described in the product manual. Then, 10^5 cells were plated per well in six-well tissue culture plates (BD Biosciences, San Jose, California). The cells were treated with doxorubicin hydrochloride at concentrations ranging from 10 nM to 5 μM in standard growth media. Control cells that were either stained with CellTrace Violet only or unstained were plated at the same time. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and the media was replaced with fresh growth media. After an additional 3 days, the cells were trypsinized and prepared for flow cytometry. Flow cytometry was performed using a BD FACSCanto II. The doxorubicin signal was measured using excitation with a 488 nm laser and detection with a 585 ± 42 nm filter. The CellTrace Violet signal was measured using excitation with a 405 nm laser and detection with a 450 ± 50 nm filter. We collected data for 10,000 cells (gated based on forward and side scatter) per condition, unless there were an insufficient number of cells remaining.

Treatment Length Study

For the dosing time study, MDA-MB-231 cells were plated as described above in section *Assay Set-Up*. Initially, the media either had a medium (0.3 μM) or high (5 μM) dose of doxorubicin. The cells were washed at various time points (12, 24, 48, 72, and 96 h) and the media was replaced with fresh growth media. All cells were read on the flow cytometer at the same time after a total of 4 days after plating.

Verapamil Treatment

Using HCT-15 cells, the study with verapamil treatment was set up as described above in section *Assay Set-Up* with 20 μM verapamil in the growth media. The replacement media after 24 h also contained 20 μM verapamil.

Data Analysis

The raw flow cytometry data were processed in the following manner in order to draw together the results from numerous single-cell measurements from different treatment conditions. FlowJo software (FlowJo, LLC, Ashland, Oregon) and MATLAB (Mathworks, Natick, Massachusetts) were used for data processing. First, the doxorubicin signal was calibrated as described in section *Calibration of Doxorubicin Signal*. Next, we normalized the CellTrace signal with respect to the median CellTrace Signal for untreated cells as described in (1).

$$\text{Proliferation factor} = \frac{\text{Median CellTrace signal of untreated cells}}{\text{CellTrace signal of sample}} \quad (1)$$

Note that the fluorescence signal from untreated cells is in the numerator of the expression in (1). Cells that did not proliferate at all have a high CellTrace signal because the CellTrace has not been diluted by growth. Thus, the proliferation factor is low for cells that had fewer cell divisions and is equal to one if cells were unaffected by treatment. The theoretical minimum for the proliferation factor with complete inhibition of growth is 2^{-n}, where n is the number of doublings for untreated cells.

With both fluorescence signals converted, the cells were binned based on amount of intracellular doxorubicin. One hundred bins were used with even logarithmic spacing from 10^4 to 10^{10} intracellular doxorubicin molecules. Any bin with fewer than 100 cells was omitted. For each bin of cells, median proliferation factor was plotted versus the median number of intracellular doxorubicin molecules resulting in a cellular response curve to doxorubicin treatment. The included plots show cellular response curves for either individual treatment conditions or for data from all treatment conditions concatenated into one response curve. When processing the fluorescence signal from control cells, half of the untreated cells appear as if they have doxorubicin signal despite never being treated with doxorubicin as the median doxorubicin signal for untreated cells was used to subtract out background fluorescence signal. In addition, the lower half of the control cells appear to have a negative number of doxorubicin cells based on the calibration of the doxorubicin signal and thus do not appear in the analysis plots as the plots are log based.

Calculation of Standard and Single-Cell IC_{50}

The standard IC_{50} is the media doxorubicin concentration required for 50% of maximum reduction in proliferation factor. The single-cell IC_{50} is the number of intracellular doxorubicin molecules required for a 50% of maximum reduction in proliferation factor. The IC_{50} values were calculated from a nonlinear regression with the "log(inhibitor) versus response (three parameter)" equation in GraphPad Prism software (GraphPad Software, Inc., La Jolla, California). Median proliferation factor for each treatment condition was used for standard IC_{50} and the median values from bins for the concatenated data for each cell line were used for single-cell IC_{50}. Confidence intervals were also calculated in the GraphPad Prism software.