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A rapid survival assay to measure drug-induced cytotoxicity and cell cycle effects

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ABSTRACT

We describe a rapid method to accurately measure the cytotoxicity of mammalian cells upon exposure to various drugs. Using this assay, we obtain survival data in a fraction of the time required to perform the traditional clonogenic survival assay, considered the gold standard. The dynamic range of the assay allows sensitivity measurements on a multi-log scale allowing better resolution of comparative sensitivities. Moreover, the results obtained contain additional information on cell cycle effects of the drug treatment. Cell survival is obtained from a quantitative comparison of proliferation between drug-treated and untreated cells. During the assay, cells are treated with a drug and, following a recovery period, allowed to proliferate in the presence of bromodeoxyuridine (BrdU). Cells that synthesize DNA in the presence of BrdU exhibit quenched Hoechst fluorescence, easily detected by flow cytometry; quenching is used to determine relative proliferation in treated vs. untreated cells. Finally, this assay can be used in high-throughput format to simultaneously screen multiple cell lines and drugs for accurate measurements of cell survival and cell cycle effects after drug treatment.

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1. Introduction

Survival of cells upon exposure to toxic agents is an important phenotypic measure used to understand the biological importance of certain proteins and pathways in either preventing or enabling cell survival after toxic stress. For example, key proteins involved in DNA repair or the DNA damage response have been identified by measuring the effect of silencing or over-expressing these proteins on cell survival after DNA damage. The gold standard for assessing the survival of cells after drug treatment in such experiments continues to be the clonogenic survival assay that is extremely sensitive and has a dynamic range of several orders of magnitude. Unfortunately, it suffers from being very low throughput as well as time and labor intensive. Typically, the clonogenic survival assay takes 10–14 days and requires a large number of cell culture plates, thus limiting its practical application to a few cell lines and to a limited number of doses or agents. Moreover, for cell lines grown in suspension, or for those that fail to form colonies, the clonogenic survival assay is done by either monitoring growth from single cells or following their ability to form colonies in soft agar. These approaches are even more time intensive taking up to 2–3 weeks to complete a single experiment. Currently, the only available high-throughput techniques for measuring sensitivity involve the correlation of viability to membrane permeability (trypan blue or propidium iodide exclusion) or measurement of metabolic activity (e.g. the MTT assay). Unfortunately, metabolic activity primarily reflects mitochondrial function; in addition changes in metabolic activity do not always correlate well with cell viability after treatment, and do not differentiate between cytotoxic and static effects after treatment. Similarly, membrane permeability only takes into account cells that undergo cell death after treatment and fails to identify sensitivity due to activation of a static program such as arrest or senescence [1]. Perhaps more importantly, these methods have an inherently limited dynamic range for detection of sensitivity, generally less than a single order of magnitude vs. three or four orders of magnitude for the clonogenic survival assay.

In this paper, we describe a rapid method for measuring the drug sensitivity of cells with a dynamic range comparable to that of the clonogenic survival assay. This assay has a much higher throughput compared to traditional clonogenic survival assays. In addition to obtaining survival information, it can also be used to deduce cell cycle effects of drug treatment. This method is based on the fact that

Abbreviations: PI, propidium iodide; CEN, chicken erythrocyte nuclei; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.

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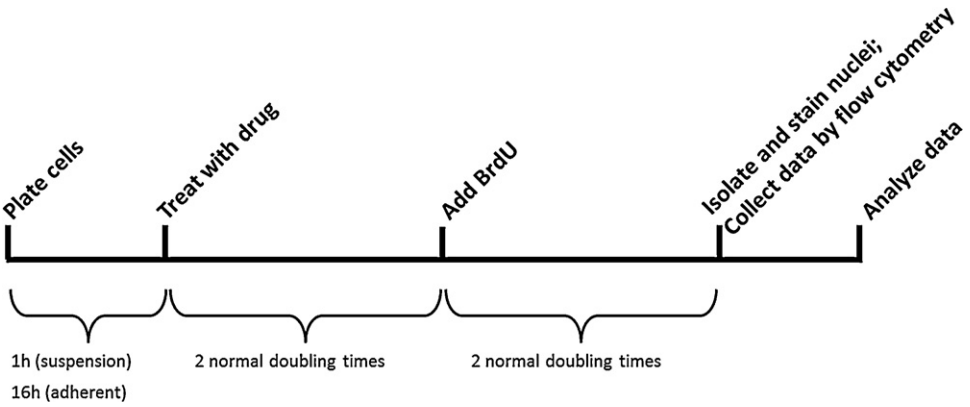

Fig. 1. Timeline of key steps in the experimental procedure.

the fluorescence of Hoechst, a dye that preferentially binds AT-rich regions in the DNA, is quenched when bromodeoxyuridine (BrdU), a thymine analog, is incorporated into DNA [2,3]. Cells that have divided zero, one or two times in the presence of BrdU can be differentiated based on the level of quenched Hoechst fluorescence, thus giving a measure of cell proliferation [4]. In a previous report, Poot et al. [5] took advantage of the Hoechst quenching property of BrdU to measure survival of cells after exposure to a DNA damaging agent. We have extensively modified the technique to be performed in a multi-well format (96-well plate for suspension cells and 24-well plate for adherent cells), drastically decreasing the setup time and reducing the number of cells required for a survival curve to as little as 10^6–3×10^6 cells. Furthermore, we have broadened the scope of the assay so that it can simultaneously be used with different cell types and different cytotoxic agents. The assay can be completed within half the time taken to perform a clonogenic survival assay while maintaining high sensitivity and a dynamic range of three to four logs of magnitude.

Fig. 1 shows a concise representation of the steps involved in the assay. After cells are treated they are allowed to recover for the duration of two doubling times and subsequently allowed to proliferate (if they can) for the duration of another two doubling times in the presence of BrdU. To make our approach amenable to screening multiple agents with diverse mechanisms of action, we allow cells two doubling times after treatment for toxicity to present. This allows for simultaneous detection of the toxicity of agents that act immediately vs. those that require formation of intermediates to slowly build up in cells. As an example, various DNA damaging agents are dependent on replication for toxicity to occur, and several days may need to pass prior to an observable phenotypic response. The duration of time prior to BrdU addition can be optimized depending on the agents to be tested. At the end of the assay, cells are gently lysed to obtain nuclei that are stained with propidium iodide (PI) and Hoechst dye. Nuclei fluorescence is measured by flow cytometry to quantify the percentage of cells that have proliferated in the presence of BrdU. The relative proliferation rate of treated samples compared to untreated controls gives a measure of the sensitivity of cells to treatment. The ease, economy and efficiency of this assay will enable rapid progress in systematic approaches to understanding the biological importance of many proteins and pathways whose modulation leads to an observed phenotype after exposure to cytotoxic agents.

2. Materials and methods

2.1. Cell culture

The human lymphoblastoid cell lines TK6 [6] and TK6 derivatives (MT1 [7] and TK6 + MGMT [8]) were grown in suspension in RPMI medium supplemented with 10% equine serum, 1% L-glutamine and 1% penicillin–streptomycin. TK6 and MT1 cell lines were derived from the same parent and both lack the DNA repair protein MGMT whereas the TK6 + MGMT cell line contains reconstituted MGMT. A genetically diverse set of human lymphoblastoid suspension cell lines were obtained from the Coriell Institute for Medical Research and cultured in RPMI medium (Invitrogen) supplemented with 15% FBS, 1% penicillin–streptomycin and 1% L-glutamine. The cell lines were numbered 1–24 for ease of handling. The Coriell catalog numbers for the cell lines 1–24 are respectively, GM15029, GM13036, GM15215, GM15223, GM15245, GM15224, GM15236, GM15510, GM15213, GM15221, GM15227, GM15385, GM15590, GM15038, GM15056, GM15072, GM15144, GM15216, GM15226, GM15242, GM15268, GM15324, GM15386 and GM15061. The adherent U87MG glioblastoma cell line was obtained from ATCC (Rockville, MD) and grown in DMEM medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin–streptomycin.

2.2. Determining the optimal BrdU concentration

Cells are grown in the presence of different concentrations of BrdU (0–100 μM) for one doubling time. Cells are then lysed and stained as described below with Hoechst and propidium iodide for flow cytometry analysis. The optimal BrdU dose is determined as that which quenched Hoechst fluorescence of G1 cells by half after one doubling time. This dose allows the effective resolution of cells that have undergone one division after BrdU addition from those that have undergone none or two divisions after addition. This optimal dose was determined as 45 μM for the lymphoblastoid suspension cell lines and 20 μM for the U87MG cell lines.

2.3. Cell cycle profile analysis by flow cytometry

BrdU is sometimes known to cause a G2/M arrest in cultured human cells. TK6, TK6 derivatives and U87MG cell lines were grown in the presence of the optimal BrdU concentration for at least two doubling times, during which samples were collected at multiple time points, washed with cold PBS and fixed overnight in cold 100% ethanol. Fixed cells were washed with PBS + 1% BSA, resuspended in PBS + 1% BSA containing propidium iodide (50 μg/ml) and immediately analyzed by flow cytometry to obtain cell cycle profiles. None of the cell lines showed a G2/M arrest when grown in the presence of BrdU. If however such an arrest is observed, the effect can be overcome by adding deoxycytidine at an equal concentration as the added BrdU [3].

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