Short Technical Report

Thymidine Incorporation Is Highly Predictive of Colony Formation and Can Be Used for High-Throughput Screening

BioTechniques 30:1118-1124 (May 2001)

**John R. Lamb, Sondra Goehle,
Cathy Ludlow, and Julian A.
Simon**

Fred Hutchinson Cancer
Research Center, Seattle,
WA, USA

ABSTRACT

The primary goal of anticancer chemotherapy is to kill cancer cells. Therefore, it is of critical importance that any assay that is used to determine the toxicity of a potential anticancer drug accurately measures viability. While colony formation is widely regarded as the most accurate measure of viability following drug treatment, it is laborious, time consuming, and difficult to carry out with non-adherent cells. For these reasons, it is not suitable for moderate- to high-throughput screening applications. We sought to identify a convenient and reliable assay that would accurately reproduce colony formation results and be amenable to high-throughput applications. Here, we describe a modification of the ^3H -thymidine incorporation assay that meets these criteria. The assay can be carried out in 96-well plates with minimal handling of reagents and media. It can be performed with non-adherent and adherent cell lines. Most importantly, LC_{50} values obtained with this assay show excellent agreement with colony formation results. Taken together, these advantages make the modified ^3H -thymidine incorporation assay well suited for high-throughput viability assays in anticancer drug discovery and development.

INTRODUCTION

The ultimate measure of a cell's viability is its ability to form viable progeny. The colony-forming assay is an accurate measure of viability because it measures a cell's ability to undergo multiple cell divisions after treatment. In contrast, many widely used viability assays actually detect particular events associated with cell death that may not be applicable to all cell types. Apoptosis (10), for instance, reflects cell death, but the absence of apoptosis does not necessarily indicate viability. Similarly, the MTT assay (1,11) measures loss of mitochondrial function, but its presence does not necessarily indicate viability. What is needed is a toxicity assay that, like colony formation, measures the ability to grow after treatment but is adaptable to high throughput.

We propose that, since S-phase is required for growth, the ability to replicate DNA must correspond to colony-forming ability. Here, we present the results of a comparison between colony formation and ^3H -thymidine incorporation data using four human cancer cell lines and three FDA-approved anticancer agents. Incorporation of ^3H -thymidine immediately following treatment is commonly used to measure direct effects on DNA synthesis (6). In the short term, DNA synthesis is likely to be affected by damage and checkpoints and may not reflect ultimate viability. However, at some point after treatment, viable cells must reenter the cell cycle and undergo DNA synthesis. Therefore, at time points after the beginning of treatment, measurement of DNA synthesis should

correlate with viability (7). We have chosen to measure DNA synthesis by ^3H -thymidine incorporation four days or greater than three cell cycle lengths after the start of drug treatment to avoid short-term effects of cell-cycle checkpoints on DNA synthesis. The cell lines selected represent a spectrum of molecular defects, including p53 function, ras mutation, and epidermal growth factor receptor expression, as well as a variety of tumor types (Table 1). We also chose agents with different mechanisms of action and different determinants of sensitivity. In all cases, the ^3H -thymidine incorporation four days after the start of treatment shows a high degree of agreement with colony-forming results. Similar results are obtained with adherent and non-adherent DT40 cells treated with etoposide. Taken together, these results indicate that the modified ^3H -thymidine incorporation assay is a practical alternative to colony formation for high-throughput screening.

MATERIALS AND METHODS

Cell Culture

A549, ACHN, HCT116, and SW620 were provided by the Developmental Therapeutics Program of the National Cancer Institute. The xrs6 and DT40 matched pairs were obtained from P. Jeggo (MRC Cell Mutation Unit, University of Sussex, UK) and S. Takeda (CREST, Japan Science and Technology, Kyoto University, Japan). All cell lines, with the exception of the chicken cells, were grown in DMEM

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Table 1. Cell Lines Used in This Study

Cell Line	Species	Tissue	Doubling Time (hrs)	Activated ras	p53	EGF mRNA (Relative Units)	Rhodamine Efflux (Channel Shift)
A549	Human	lung (NSCL)	22.9	yes	wt	378	10
ACHN	Human	renal	27.5	no	wt	2141	120
HCT116	Human	colon	17.4	yes	wt	613	26
SW620	Human	colon	20.4	yes	mutant	not determined	31
Cell Line	Species/ Cell Type	Attached or Suspension Growth		Molecular Defect			
xrs6	CHO	adherent		mutated KU80			
xrs6/KU80	CHO	adherent		as above, +wt KU80			
DT40 cl18	chicken pre-B cell	non-adherent		wt			
DT40 #77	chicken pre-B cell	non-adherent		KU70 -/-			

The upper panel shows the four human tumors cell lines used in Figure 1. Details of the molecular targets and doubling times were taken from the Molecular Targets database of the NCI (<http://www.dtp.nci.nih.gov>). Briefly, the ras and p53 sequences were determined by sequencing PCR-amplified cDNA, EGF mRNA levels by northern hybridization, and efflux of rhodamine123 by flow cytometry. The lower panel shows the cell lines used in Figure 2 (8,9). wt, wild-type.

supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified incubator with 5% CO₂ atmosphere. The chicken cell lines DT40 cl18 and DT40 no. 77 were grown in RPMI with 10% FCS, 1% chicken serum, 10 µM 2-mercaptoethanol, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C.

Colony-Forming Assays

One hundred and 1000 cells/well are plated in six-well plates and incubated overnight. Medium containing various concentrations of the test compound is added. Each dose of compound is tested in triplicate. After a 48-h incubation, the cells are placed in fresh medium and incubated until colonies form (usually 10–20 days). Cells are stained with methylene blue, and the colonies are counted. Colony-forming ability is expressed as a percentage of the vehicle-treated controls, and each value is the average of three determinations.

³H-Thymidine Incorporation Assay

Five hundred cells/well are plated in

opaque 96-well microplates (Packard Instrument, Meriden, CT, USA) in 135 µL medium. After overnight incubation, test compounds (10×stock) are added in 15 µL medium/well. Each drug concentration is tested in triplicate. After 48 h exposure, the cells are incubated for an additional 48 h in fresh medium. The cells are then incubated in medium containing 2 µCi/mL ³H-thymidine for 24 h, washed three times with PBS, 100 µL scintillant are added, and the plates are sealed and counted on a 96-well liquid scintillation counter (TopCount®; Packard Instrument).

Non-adherent cell lines are plated at a density of 500 cells/well in clear round-bottom plates as above. After addition of the test compound, the cells are incubated for 96 h. Medium (50 µL/well) containing 8 µCi/mL ³H-thymidine is added to the drug-containing medium and incubated for 24 h. The cells are then washed with PBS onto GF/C filter plates (UniFilter™) using a plate washer (both from Packard Instrument). After the plates have dried, 20 µL scintillant are added, and the plates are sealed and counted. Toxicity is expressed as the percentage of vehicle-treated controls, and each value is the

average of three determinations.

For adherent cell lines, this assay can be performed with a conventional liquid scintillation counter as follows. Five thousand cells/well are plated in 24-well plates and treated as above with appropriately adjusted volumes. After washing, 100 µL 0.2 M NaOH are added to each well and transferred to scintillation vials. Five milliliters of scintillant are added, and the vials are counted.

Best results are obtained when the counts/well are 100-fold or greater above background (5000–10 000 cpm). For primary or slow-growing cell lines, the counts incorporated can be raised by increasing the number of cells seeded and/or the concentration and length of exposure to ³H-thymidine. For instance, with mouse embryonic fibroblasts, we seed 5000 cells/well and, after treatment, expose them to 10 µCi/mL ³H-thymidine for 96 h. We have successfully used this procedure with primary, immortal, and transformed cell lines from chicken, rat, mouse, hamster, and humans, and cell types including fibroblast, keratinocyte, and lymphocytes, and a variety of cancer lines derived from various tissues. In addition, several thousand compounds have been

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screened in matched pairs of cells, yielding compounds with differential toxicity, confirmed by colony formation.

RESULTS AND DISCUSSION

The following four human tumor cell lines were chosen: A549 (non-small cell lung), ACHN (renal), HCT116, and SW480 (colon; Table 1). Preliminary experiments showed that these cell lines had differing sensitivities to etoposide, hydroxyurea (HU), and taxol. These compounds were chosen as standard agents with differing mechanisms of action and differing effects on cell cycle progression: etoposide, a topoisomerase II poison that leads to induction of DNA double-strand breaks and a G2 arrest; HU, an inhibitor of ribonucleotide re-

ductase that causes an early S-phase arrest; and taxol, a microtubule poison that causes arrest in mitosis. In addition, we wanted to test whether ^3H -thymidine incorporation would give similar results with cytostatic and/or cytotoxic agents and, in the case of HU compounds, those that might affect thymidine incorporation.

The effect of a 48-h exposure to the three compounds on the colony-forming ability of the four cell lines was measured (Figure 1, left column), and a range of sensitivities was observed. We next assessed whether ^3H -thymidine incorporation reflects the colony-forming ability of these cells after exposure to the same compounds for the same length of time (Figure 1, right column). Overall, both the rank order of sensitivities and the degree of sensitivity for a

given dose are reproduced with ^3H -thymidine incorporation under these conditions. With ^3H -thymidine incorporation, A549 appears to be somewhat more sensitive to etoposide than is found by colony formation. This indicates that 100% predictive value of the ^3H -thymidine assay is not achieved. However, in 11 of the 12 cases, good correspondence is seen. Therefore, ^3H -thymidine is highly predictive of the colony-forming ability of these cells exposed to these compounds. At present, we do not know why A549 is more sensitive to etoposide in the thymidine assay, but this is not a general effect of the cell line because good correlations were seen with HU and taxol.

We next compared the assays in a matched pair of cells with differential sensitivity to etoposide. The pair of Chinese hamster ovary-derived cell lines used are identical apart from expression of the double-strand break repair protein KU80 (3,5,8). By colony formation, cells lacking KU80 (xrs6) are much more sensitive to etoposide than isogenic cells that express KU80 (xrs6/KU80; Figure 2, left graph). Similarly, xrs6 cells show reduced ^3H -thymidine incorporation after exposure to etoposide that is comparable to that seen with colony formation (Figure 2, left and middle graphs).

KU70 associates with KU80 and is also required for double-strand break repair (2–4). Both copies of the KU70 gene have been disrupted in the chicken pre-B cell line DT40 (9). This double-strand break repair-deficient cell line should also be sensitive to etoposide (4). Assessing toxicity in these non-adherent cell lines by colony formation is technically challenging but is relatively easy by ^3H -thymidine incorporation. Figure 2 shows that similar toxicity and differen-

Figure 1. Comparison of colony formation (left column) and ^3H -thymidine incorporation (right column) measures of toxicity. Four human tumor cell lines were exposed to the same concentrations of etoposide (top), HU (middle), and taxol (bottom) for the same length of time and processed as described in Materials and Methods. The untreated samples were adjusted to 100%, and the treated samples are expressed as a relative percentage. This adjustment accounts for differences in growth rates and allows direct comparisons between cell lines. The error bars are standard errors that incorporate the error of the untreated samples.

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Figure 2. Toxicity assays in matched pairs of adherent or non-adherent cells with and without double-strand break repair. Etoposide is selectively toxic to cells that lack expression of KU80 (xrs6) compared to isogenic cells that express KU80 (xrs6/KU80) as measured by colony formation (left graph). A similar finding is made with the ^3H -thymidine incorporation assay (middle graph). The non-adherent chicken pre-B cell line DT40 disrupted for the double-strand break repair protein KU70 (which forms a heterodimer with KU80) is also sensitive to etoposide relative to isogenic repair proficient cells as measured by ^3H -thymidine incorporation (right graph).

tial toxicity is seen with DT40 cells with and without KU70, in comparison to xrs6 cells with and without KU80.

In conclusion, ^3H -thymidine incorporation, four days after the start of exposure to anticancer agents, appears to be highly predictive of colony formation in a variety of cell types. We have successfully used this assay on a wide variety of cell lines, including primary and immortalized cells from human, hamster, mouse, rat, and chicken. In a pilot experiment, we used this assay to screen for compounds selectively toxic to double-strand break repair-deficient cells. This led to the identification of two novel topoisomerase II poisons (manuscript submitted). Furthermore, we have used this assay to screen several thousand compounds for differential toxicity in rat fibroblasts with and without c-Myc overexpression and identified several lead compounds. In both screens, large numbers of compounds were tested over a range of concentrations, proving the applicability of the ^3H -thymidine assay to high-throughput applications.

The ^3H -thymidine assay described here is simple and easy to perform, has a seven-day turnaround, is applicable to both adherent and non-adherent cells, is relatively high throughput, and, most importantly, is highly predictive of colony-forming ability. This assay is widely applicable, highly predictive, and can be used as a high-throughput test for drug discovery.

ACKNOWLEDGMENTS

We would like to thank Lee Hartwell and Stephen H. Friend; Priscilla Cooper, Stephen J. Elledge, Jan Hoeijmakers, Andrew Murray, Tom Petes, and Graham C. Walker for invaluable advice; and Edward Sausville and members of the Developmental Therapeutics Program of the National Cancer Institute for many helpful comments and advice. This work was supported in part by contract no. NO1-BC65017 (J.A.S.) and also by grant no. 1 RO1 CA82311-01 (J.R.L.) both from the National Cancer Institute.

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Received 13 February 2001; accepted 23 March 2001.

Address correspondence to:

Dr. John R. Lamb
Program in Molecular Pharmacology
Clinical Research Division
Fred Hutchinson Cancer Research Center
Mailstop D2-100
1100 Fairview Ave.
N. Seattle, WA 98109, USA
e-mail: jlamb@fhcrc.org