A RAPID AND SIMPLE MTT-BASED SPECTROPHOTOMETRIC ASSAY FOR DETERMINING DRUG SENSITIVITY IN MONOLAYER CULTURES

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SUMMARY: A rapid and sensitive spectrophotometric assay for determining viability in monolayer cultured cell lines, with specific applications in normal and drug resistant cell line determinations, is described. The assay involves conversion of the tetrazolium salt MTT by viable proliferating cells to an insoluble product, purple formazan. The chief advantage of this assay is that it requires fewer cells than standard cytotoxicity assays. In addition, it allows for multiple sample concentrations on a single 96-well plate which is then rapidly quantitated using an automated spectrophotometric microplate reader.

Key words: MTT; tetrazolium salt; spectrophotometric; monolayer; drug resistance; cytotoxicity; proliferation.

I. INTRODUCTION

This report describes a simple, reproducible spectrophotometric assay using MTT to determine both cell viability and proliferation in drug-sensitive and resistant cell lines. The assay utilizes a tetrazolium salt, MTT, which is converted by mitochondrial dehydrogenases in viable cells to an insoluble product termed purple formazan. MTT labeled cells are lysed using an acidified isopropanol solution, and the plate is rapidly read with an automated spectrophotometric plate reader. The chief advantages of this method are the ability to assay using fewer cells than standard cytotoxicity assays and to test replicate samples over a wide range of drug concentrations using a single assay plate. Standard methods of assessing cell viability or proliferation or both have involved: a) the exclusion of dyes such as trypan blue, b) measurements of the release of radiolabeled compounds such as ^{51}Cr after cell lysis, or c) measurements of the incorporations of radioactive nucleotides such as [^{3}H]thymidine. The availability of a rapid, simple nonisotopic assay that assesses the functional state of the cellular energy source, i.e. the mitochondria, has wide applications for both cytostatic and cytotoxic testing.

II. MATERIALS

A. Chemicals
1. Isopropanol (2-Propanol), A-416, Fisher Scientific[1]
2. 1 *N* HCl, SO-A-48[1]
3. MTT (3,(4,5-dimethylthiazoyl-2-yl) 2,5 (diphenyl-tetrazolium bromide), M-2128, Sigma[2]

B. Cells and cell culture reagents
1. Phosphate-buffered saline (PBS), pH 7.2, 450-1300EB, GIBCO[3]
2. Trypsin (0.05%), 1:250 in 0.53 m*M* EDTA, 840-7250[3]
3. Trypan blue, 0.4%, 630-5250[3]
4. Dulbecco's modified Eagle's medium (DME), 430-1600[3]
5. Gentamicin (25 μg/ml), 320-1885AJ[3]
6. Fetal bovine serum (10%), A-115-L, HyClone[4]
7. SV40-transformed WI-38 cells, AG7215, NIA Aging Cell Culture Repository[5]

C. Plastics and glassware:
1. Pipette tips, sterile, 77-984-H2, Flow[6]
2. 100 × 15-mm² disposable culture dishes (Lab Tek 4021), 245387-024, VWR[7]
3. Pasteur pipettes, sterile, unplugged, 13-678-20D[7]
4. Tubes, plastic, sterile, 12 × 75, 60819-310[7]
5. 96-well tissue culture plates, 3596, Costar[8]

D. Equipment
1. Vortex-Genie, 12-812[1]
2. Flow MCC Multiskan ELISA Reader, equipped with 570 nm wavelength filter, 78-504-00[6]
3. Titertek Repeat Pipetter, 77-829-00[6]
4. SarVac vacuum aspirator pump, Sargent Welch model 8803B, 54969-828[7]
5. Air/CO_2 incubator, model 3028, Forma Scientific[9]

III. PROCEDURE

A. Preparation of cells for plating
1. Trypsinize cells and perform viability count.
2. Resuspend cells to give a total of 2 × 10^5 viable cells in 10 ml of the appropriate growth medium.

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3. Mix well and transfer the cell suspension to a sterile, square dish.
4. Using the repeat pipetter, plate 100-μl/well into columns 2-10 of a 96-well plate for a final concentration of 2×10^3/well.
5. Fill columns 1 and 12 with 200 μl of medium without cells to retard evaporation.
6. Place the plate in a humidified incubator at 37° C for 24 h to allow the cells to attach.

B. Preparation and addition of drugs or reagents for testing
1. Prepare 2× concentrations in medium of the appropriate drug to which the cells are to be tested for sensitivity. Duplicate columns can be run as negative controls to ensure that well location is not a factor in the assay. Columns 2 and 7 are routinely used by our lab as negative control columns.
2. Add 100 μl of the appropriate concentrations to all eight wells of the designated columns and return the plate to the incubator for the desired incubation period.
3. For pulse treatment, the wells are gently vacuum aspirated using a sterile Pasteur pipette for each column to avoid cross contamination. The wells are washed with 200 μl of warm medium for 10 min. The wash medium is aspirated, the wells are then replated with fresh medium, and the plate is returned to the incubator for a 4-d growth period.
4. Assays requiring continuous treatment are grown for 5 d in the presence of the drug or reagent.

C. Preparation of stock MTT
1. Weigh out 15 mg of powdered MTT per plate to be assayed.
2. Dissolve the MTT in 3 ml of warm PBS in a disposable plastic tube wrapped with foil to protect from light. Vortex the tube vigorously to ensure that all of the MTT goes into solution. Filtering is generally not necessary.

D. Addition of stock MTT to assay plates
1. Using the repeat pipetter, add 20 to 25 μl of stock MTT to each well of the plate to include columns 1 and 12. These will serve as blanking columns to determine nonspecific reaction of the MTT with the assay plate.
2. Incubate the plate for 4 h at 37° C in the dark.

E. Preparation and addition of acid-isopropanol
1. Prepare 10 ml of acid-isopropanol solution per plate to be assayed by combining 9.6 ml 2-Propanol with 0.4 ml of 1 *N* HCl. Final concentration is 0.04 *N* HCl in isopropanol.
2. Carefully vacuum aspirate all wells of the assay plate.
3. Invert the plate to ensure that all MTT solution has been removed. *Note*: Photos of the wells may be made at this time.
4. Using the repeat pipetter, add 100 μl of acidified-isopropanol solution to each well of the assay plate.
5. Cover and gently tap all four sides of the plate to ensure complete solubilization of the purple formazan.

F. Plate reading and calculations
1. The plate is read using the single wavelength mode with a 570-nm wavelength filter. Blanking is performed on column 1, or column 12 if the plate is to be run in the reverse direction. It is recommended that the plate be read in both directions to ensure accuracy and reproducibility of the assay.
2. Means or standard deviations or standard errors or both are then calculated for each column.
3. Cell growth expressed as a percent of control is calculated as:

$$\frac{\text{mean of test wells}}{\text{mean of control wells}} \times 100$$

4. Percent cytotoxicity is calculated as:

$$1 - \frac{\text{mean of test wells}}{\text{mean of control wells}} \times 100$$

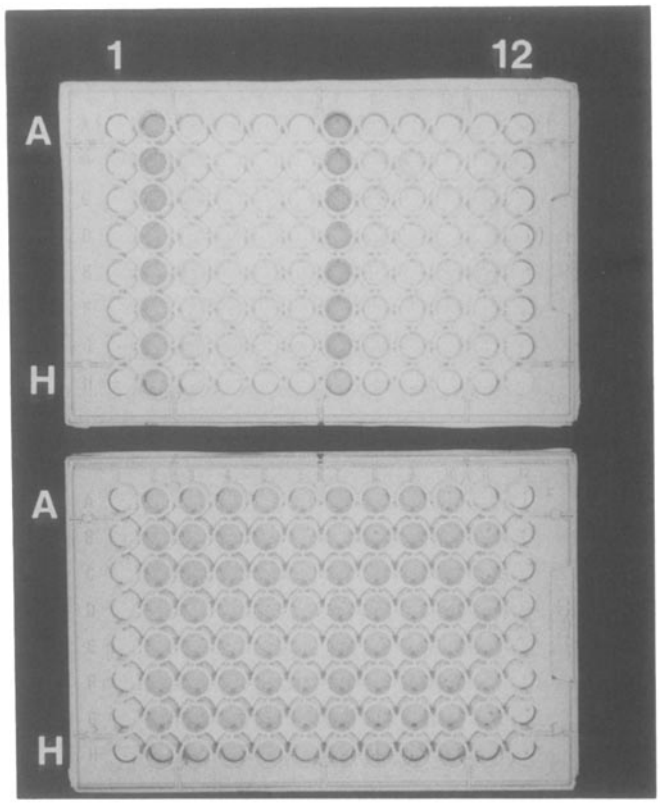

FIG. 1. Comparison MTT assays of wild-type (*top*) and 20 μg/ml tunicamycin resistant (*bottom*) SV40-transformed WI-38 lung fibroblasts. Cells were seeded at 2×10^3/well and cultured for 5 d in the presence of various concentrations of tunicamycin. Twenty-five microliter of a 5 mg/ml stock MTT solution was added for 4 h, and the cells lysed using 100 μl of 0.04 *N* HCl in isopropanol. The plates were stored sealed in foil at −20° C until photographed. Tunicamycin concentrations: columns 2 and 7, 0 μg/ml; columns 3 and 8, 0.5 μg/ml; columns 4 and 9, 1 μg/ml; columns 5 and 10, 10 μg/ml; columns 6 and 11, 20 μg/ml; columns 1 and 12, blank.

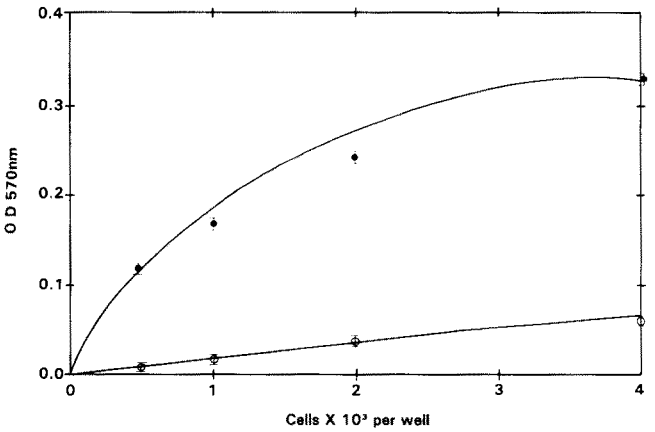

FIG. 2. Graph of SV40-transformed WI-38 cells seeded at varying cell concentrations and grown for 5 d alone (— ● —) or in the presence of 0.5 μg/ml mitomycin C (—○—). Twenty-five microliter of a 5 mg/ml stock MTT in PBS solution was added to each well and incubated for 4 h. The wells were aspirated and the cells lysed using 100 μl of 0.04 *N* HCl in isopropanol. The plate was read at 570 nm on a Flow Multiskan MCC plate reader. *Solid lines* represent the best fit approximation of each set of experimental data; *circles* represent the original experimental points; *bars* indicate mean ± SEM.

IV. DISCUSSION

We have used the above-described procedure to assay virally transformed lung fibroblasts which have been stepwise selected for resistance to the glycosylation inhibitor tunicamycin and to determine cross-resistance to other drugs such as 2-deoxy-D-glucose, actinomycin D, and mitomycin C (1). A comparison of the results of an MTT assay can be seen in Fig. 1. SV40-transformed WI-38 lung fibroblasts, which had been stepwise selected for resistance to 20 μg/ml tunicamycin, were tested concurrently with wild-type cells in the presence of various concentrations of the drug. Note that the total number of cells required for an entire MTT assay plate is only twice that required for a single 60-mm dish used in standard cytotoxicity assays. For assessments of cell viability alone, 0.1 to 0.5 μg/ml mitomycin C pretreatment of target cells serves to block cellular replication before treatment with the agent of interest. A comparison graph of the proliferative ability, as assessed by MTT assay, of varying concentrations of SV40-transformed WI-38 cells seeded alone or in the presence of 0.5 μg/ml mitomycin C can be seen in Fig. 2.

The chief advantage of the MTT assay over other rapid dye assays such as the neutral red spectrophotometric assay (2) is that it assesses viability and proliferation based on the functional state of the cell mitochondria (3). The versatility of the MTT assay has been demonstrated in applications for the quantitation of cytotoxic and growth inhibitory lymphokines (4) and for the isolation and characterization of antiproliferative cell surface directed monoclonal antibodies (5). This versatility allows for the differentiation between cytostatic and cytotoxic monoclonal antibodies. Its simplicity, reproducibility, economy, and versatility make the MTT assay a most advantageous one for toxicity testing and for routine cell culture applications.

V. REFERENCES

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