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Evaluation of the Cytokinesis-Block Method for Assessing Genotoxicity

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Abstract

Controversy exists on whether to use cytochalasin B (Cyt-B) when analyzing genotoxic agents with the micronucleus assay. Cyt-B will block cells in a multinucleate phase, making it easier to evaluate micronucleus formation and assess early-stage cytotoxicity. However, there is some concern that this method may not give correct micronucleus frequencies and test their compounds with mononucleate cells (without Cyt-B).

The Cellomics® Micronucleus BioApplication and HitKit® HCS reagent kit were used to first evaluate the toxicity of Cyt-B and the Cellular Dye component. Suggested concentrations and incubations were not found to cause genotoxicity/cytotoxicity in either component. The kit was then used to compare other compounds with and without Cyt-B in CHO-K1 cells. Micronucleus frequencies were similar using either procedure.

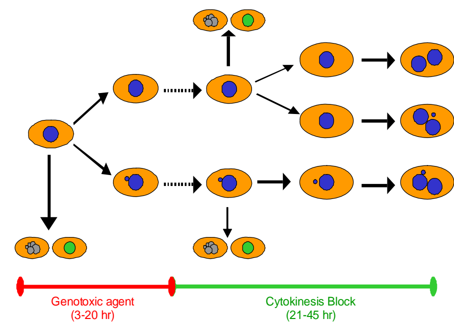
Results for both early- and late-stage cytotoxicity can be calculated when using Cyt-B with this BioApplication. Only late-stage toxicity can be evaluated with mononucleate cells. Therefore, using the block method may improve compound evaluation for early toxicity.

Introduction

Micronucleus (MN) formation may result from clastogens, which cause chromosomal breaks, and/or aneugens, which affect the spindle apparatus and result in the loss of whole chromosomes during anaphase (Maier and Schmid 1976; Hayashi et al. 1984). The *in vitro* micronucleus assay is among a new set of genetic toxicology assays wherein cultured cells are treated and scored for micronucleus induction. This assay employs cultures of established cell lines, cell strains, or primary cell cultures, where they are selected on the basis of their growth ability in culture and their spontaneous micronucleus frequency.

Analysis of the induction of micronuclei in human lymphocyte cultures has indicated that the most convenient stage to score micronuclei is during the binucleate interphase stage (Fenech and Morley, *Cytobios,*1985). Such cells have completed one cell division after chemical treatment and are therefore capable of expressing micronuclei. Treatment of cultures with the inhibitor of actin polymerization, cytochalasin B (Cyt-B), prevents cytokinesis, resulting in the trapping of cells at the binucleate or multinucleate stage where they can be easily identified (Fenech and Morley, *Mut. Res,* 1985). The measurement of the relative frequencies of multinucleated to mononucleated cells within a culture also provides a simple method of measuring the toxicity of a treatment, where toxic treatments that affect cell cycle progression will result in fewer multinucleate cells (Fenech, 1997). Figure 1 gives a visual representation of this process.

Figure 1. Principle of micronucleus induction with the Cytokinesis-Block Method



Initially cells, upon treatment with a genotoxic agent, may become apoptotic (multiple grey nuclei), cytotoxic (green nuclei), or form spontaneous micronuclei. Upon addition of cytochalasin B, cells may become apoptotic, cytotoxic, be blocked in cytokinesis (blue binucleated cells), or be blocked in cytokinesis with the induction of micronuclei.

However, there are those who would rather avoid the possibility of additional treatment altering their results and instead count micronuclei in mononucleate cells. The use of Cyt-B in experimentation has been a continued topic of discussion. Avoiding the blocking process reduces the total experimental time, but if analyzing through visual representation, would still be time-consuming. Also, it would be difficult to determine if a micronucleus formed due to compound treatment, or if other cellular stresses occurred prior to addition of an agent.

The ultimate value of the micronucleus *in vitro* assay is to screen for indicators of genetic toxicity earlier in the development of therapeutic candidates, which will impact attrition rates and yield substantial financial gains. Many of these assays are time-consuming, require multiple trained personnel, can be subjective, and are not automated, with conventional using automated analysis. The Cellomics MicroNucleus BioApplication provides quantitative information on genotoxicity (micronucleus formation) and cytotoxicity, with specific parameters to allow selection of the targeted population (number of nuclei/cell, micronuclei characteristics, etc). Using the Micronucleus Reagent Kit helps to easily perform the biological test, providing stains to label nuclei and micronuclei, cytoplasm, and cells with increased membrane permeability, as well as, if preferred, to block the cells in cytokinesis.

Therefore, the goals for this poster are:

- To determine if Cyt-B used in the assay is genotoxic
- To determine if the Cellular Dye used with the Micronucleus Reagent Kit is toxic
- To compare results between mononucleate (no Cyt-B) and binucleate (Cyt-B added) assay protocols and to determine the genotoxicity of specified compounds

Materials and Methods

Materials

Cells and Plates: CHO-K1 cells (ATCC, Manassas VA) were passaged at 70% confluency in complete F12K media (Hyclone, Logan UT) (complete = Heat-Inactivated FBS, Pen-Strep, and L-Glutamine). Cells were plated onto Collagen-I coated 96-well microplates (Becton Dickinson, Franklin Lakes NJ) to ensure attachment and were ~70% confluent at the time of fixation.

Compounds and Stains:

- **Micronucleus HitKit HCS Reagent Kit** (Cellomics) was used for all experiments (staining and cytokinesis blocking) – Cellular Dye (cytoplasm), Permeability Dye (membrane permeability), Hoechst Dye (nucleus and micronucleus), Cytokinesis Blocking Agent, and wash buffer.
- Compounds selected were the following:
  - **Mitomycin C** (MMC, EMD, San Diego CA) - inhibits DNA synthesis by cross-linking DNA
  - **Bleomycin sulfate** (Bleo, EMD) - mixture of cytotoxic glycopeptides that inhibits DNA synthesis by reacting with DNA and causing strand scission
  - **Cytarabine** (Ara-C, Sigma, St. Louis MO) - inhibits DNA synthesis by impeding chain elongation by DNA polymerase
  - **Mechlorethamine hydrochloride** (Mech, Sigma) - nitrogen mustard forming inter– and intra-strand crosslinks with DNA
  - **Cytochalasin D** (Cyt D, Sigma) - inhibits cytoplasmic division by blocking the formation of contractile microfilaments
  - **Phorbal-12-myristate-13-acetate** (PMA, EMD) – PKC activator and potent skin tumor promoter
  - **Chlorambucil** (Chl, Sigma) - anticancer drug that alkylates DNA and induces apoptosis
- Fixation was by using Formaldehyde (Fisher Scientific, Fair Lawn NJ) diluted to 3.7%

Methods

Toxicity Test: Cytochalasin B

CHO-K1 cells were plated at 3000 cells per well and incubated at 5% CO_2 for 18-22 hr. Media was removed and Cellular Dye was added to all wells and incubated for 1 hr. Dye solution was removed and cells were washed once with media. A dose-response of Cyt-B (0.375 - 24 µg/mL) was added to appropriate wells and incubated for different amounts of time (6 – 48 hr). All doses were done in triplicate for each time point. Permeability Dye was added to all of the wells 30 min prior to fixation. Solution was removed and cells were washed once with media. Media was removed and cells were fixed with 3.7% formaldehyde/Hoechst solution. Cells were washed twice with Wash Buffer-M and run on the ArrayScan with the Micronucleus BioApplication, setting the minimum and maximum nuclear count at 1 and 2, respectively.

Toxicity Test: Cellular Dye

CHO-K1 cells were plated at 2000 cells per well and incubated at 5% CO_2 for 18-22 hr. Media was removed and a dose-response of Cellular Dye was added to appropriate wells for 1 hr. Solution was removed and cells were washed with media. Media was added to all wells and incubated for 24 hr (mononucleate comparison) or 20 hr with a 28 hr Cyt-B incubation (binucleate comparison). Permeability Dye was added to all plates 30 min prior to fixation. Procedure continued as above except for setting the min and max nuclear count (1 and 2, respectively for mononucleate testing or 2 and 2 for binucleate testing).

Micronucleus Assay:

The Micronucleus Reagent Kit was used for both the mononucleate and binucleate assay. Table 1 explains the differences in procedure between the two assays. In general, CHO-K1 cells were plated on collagen-I 96 well plates and incubated at 5% CO_2 for 18-22 hr. Media was removed and Cellular Dye was added to all wells and incubated for 1 hr. Dye solution was removed and cells were washed once with media. Various doses of compounds were added and returned to the incubator for a specified period of time (20 or 24 hr). For the binucleate assay, plates were washed with media, Cyt-B (6 µg/mL) was added to all wells, and plates were returned to the incubator for 28 hr.

Micronucleus Assay, Cont'

Permeability Dye was added to all of the wells 30 min prior to fixation. Solution was removed and cells were washed once with media. Media was removed and cells were fixed with 3.7% formaldehyde/Hoechst solution. Cells were washed twice with Wash Buffer-M and run on the ArrayScan, setting the minimum and maximum nuclear count according to the assay.

Table 1. Overview of the Micronucleus Assay

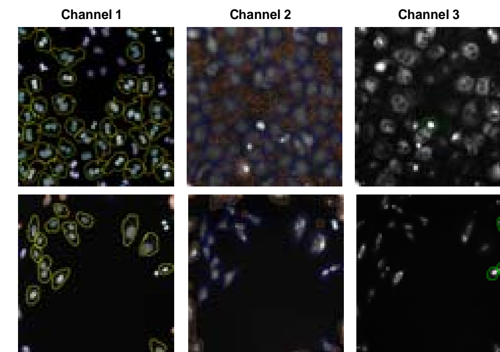
Table represents general overview of the assay and compares differences/similarities when using the mononucleate and binucleate assays.

Conditions	Micronucleus Protocol: Mononucleate Assay	Micronucleus Protocol: Binucleate Assay
Seeding Density (96-well plate)	3000 cells/well	2000 cells/well
Cellular Dye Incubation	1 hr	1 hr
Compound Incubation	24 hr	20 hr
Cyt-B Incubation	none	28 hr
Permeability Dye Incubation	30 min before fixation	30 min before fixation
BioApplication Analysis (nuclear count)	cells with one or two nuclei	cells with two nuclei

Cellomics Micronucleus BioApplication

This BioApplication (when used in conjunction with a Cellomics ® HCS Reader and Micronucleus Reagent Kit) can identify micronucleus induction in any population of cells, as well as identify agents that permeabilize the cell membrane. The algorithm has three core channels that measure the nuclei and micronuclei, cytoplasm, and cells with increased permeability respectively. Additional target channels have the capability for subpopulation analysis. There are multiple user-defined parameters to help determine the population for analysis, such as the total number of nuclei within intact cells, if nuclei are of equal size (for binucleate cells), removal of mitotic cells from analysis so that spindle fibers are not falsely identified as micronuclei, and analysis of cytotoxicity in cells with increased permeability (as well as excluded/included for calculating micronucleus frequency). Multiple output features can help determine whether the data representing the total micronucleus frequency is what is expected based on total number of cells (targeted, mononucleate, binucleate, and multinucleate), ratio of multinucleate to mononucleate cells, and overall cytotoxicity, as well as morphological changes (such as nuclear area). Figure 2 is an example of the three channels with cells treated with Cyt-B (top) or without Cyt-B (bottom).

Figure 2. Identification of Channels and Overlays using the Cellomics Micronucleus BioApplication



Images represent all channels and overlays (A) with and (B) without Cyt-B treatment.

Channel 1: **TargetedCell** (yellow), **ChosenNucCount** (teal), **Micronuclei** (pink), **ValidNuclei** (blue), and **RejectedNuclei** (orange)

Channel 2: **ValidCell** (blue) and **RejectedCell** (orange)

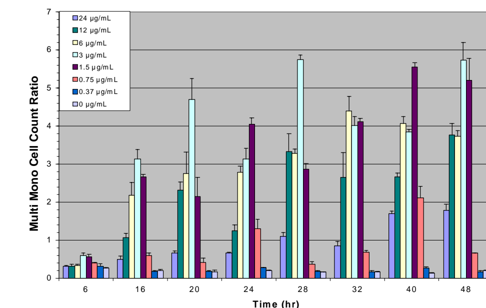
Channel 3: **CytotoxicCell** (green) based on increased membrane permeability

Results

Toxicity of Cytochalasin B

Dose-responsiveness of Cyt-B over 8 different timepoints were evaluated. The only increase in micronucleus frequency (3-fold difference over basal levels) occurred at 48 hr with concentrations at and above 3 µg/mL. When evaluating the production of binucleate cells, one can see that the use of 24 µg/mL and 12 µg/mL Cyt B causes a decrease in the number of multinucleate cells and an increase in the number of mononucleate cells (represented by the Multi Mono Cell Count Ratio output feature), a sign of early toxicity. Best concentrations/time with low micronucleus frequency and high number of multinucleate cells appeared at 3 and 6 µg/mL for 28 hr (Figure 3).

Figure 3. Dose-Responsiveness of Cyt-B Over Time

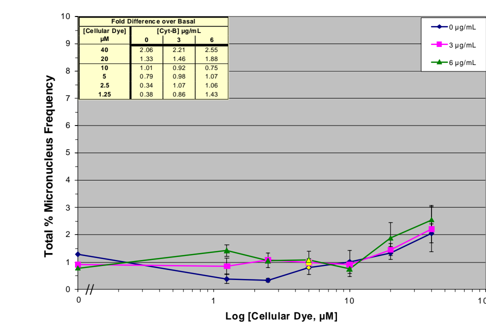


An 8-point dose response curve of Cyt-B was evaluated over eight different time points. Doses were done in triplicate and error bars represent standard deviation. Data was run with the Micronucleus BioApplication, set to evaluate both mononucleate and binucleate cells for micronucleus frequency.

Toxicity of Cellular Dye

The Cellular Dye supplied with the Cellomics Micronucleus Reagent Kit was tested at varying doses for toxicity. Figure 4 shows the percent micronucleus frequency of the doses used. Note that the concentrations with a yellow point represent the concentration suggested in the kit. Although 40 µM shows some form of toxicity (and was found to be statistically significant from a one-way ANOVA), the overall targeted cell count is less than 1000 for the mononucleate cells and less than 200 for the binucleate cells. All other concentrations were not found to be statistically significant. Inset represents the fold difference over basal levels when evaluating micronucleus frequency.

Figure 4. Genotoxicity Assessment of Cellular Dye



[Cellular Dye] µM	[Cyt-B] µg/mL: 0	3	6
40	2.06	2.21	2.55
20	1.33	1.46	1.88
10	1.01	0.92	0.75
5	0.79	0.98	1.07
2.5	0.34	1.07	1.06
1.25	0.38	0.86	1.43

A 6-point dose response curve was done in duplicate with and without Cyt-B. Error bars represent standard deviation. Data points in yellow represent the concentration of Cellular Dye suggested for use in the Cellomics Micronucleus Reagent Kit.

Genotoxicity Comparison of Compounds with and without Cyt B

Seven compounds were analyzed with the micronucleus assay, both with and without Cyt-B. Assay parameters were set so that the following analysis was performed:

- Nuclei were of correct number per cell, are within intact cells, and were of equal size and intensity (if binucleate)
- Mitotic cells were excluded from analysis
- Cells with increased membrane permeability were calculated
- Micronuclei were of correct size (1/3 or less the size of its nucleus), intensity, number, and distance

Five separate output features were observed for determining if a compound was genotoxic. Values in italics after the feature indicate the hit criteria:

1. **Targeted Cell Count** *(≥1000)*– number of cells after applying all selected criteria that limit the cells of interest .
2. **Percent Micronucleus Frequency** *(3-fold over basal)* – percent of Targeted Cells with at least one micronucleus. Statistics such as t-tests or one-way ANOVA tests may be used to determine if values are significant over basal levels (e.g., p ≤ 0.05).
3. **Multinucleate to Mononucleate Cell Count Ratio** *(above 1)* – ratio of selected cells that have more than one selected nucleus versus selected cells that have only one selected nucleus. This can be used as an early indicator of cytotoxicity, reporting on potential cell-cycle delay.
4. **Cytokinesis Block Proliferation Index (CBPI)** *(1.8-2.1)* – used to determine the number of cell cycles per cell during the period of exposure to Cyt-B (Kirsch-Volders et. al; 2003, OECD Guidelines, 2004). This is calculated as:

$$\frac{\#\ Mononucleate\ cells + 2(\#\ Binucleate\ cells) + 3(\#\ Multinucleate\ cells)}{Total\ \#\ cells}$$

   A value of 1 corresponds to all mononucleate cells and 100% cytotoxicity. Another means of calculating cytotoxicity may be done through the equation below:

$$\%Cytotoxicity = 100-(100*((CBPI_{treated}-1)/(CBPI_{control}-1)))$$

5. **Percent Cytotoxicity for Cells in Ch3** *(below 50%)* – percent of selected cells that have the maximum intensity within the nuclei above a defined threshold (based on membrane permeability). This can be used as an indicator of late-stage toxicity.

Overall, all compounds except Cyt-D and PMA met the hit criteria and were found to be genotoxic in both mononucleate and binucleate experiments. Concentrations found to be genotoxic had DMSO values below toxic levels and were not found to be statistically significant (data not shown). The table below shows both the lowest effective dose (LOED) determined from the above guidelines and the LOED determined by comparing each dose to the basal levels and performing a one-way ANOVA.

Table 2. Comparison of Compounds with and without Cyt-B

Compound	LOED Cx "hit" — Cytochalasin B (µg/mL): 0	LOED Cx "hit": 6	LOED One-way ANOVA: 0	LOED One-way ANOVA: 6
Mechlorethamine	0.019	0.019	0.009	0.009
Mitomycin C	0.156	0.039	0.019	0.009
Cytarabine	0.62	0.15	0.078	0.078
Chlorambucil	6.25	3.125	3.125	1.56
Bleomycin	1.875	0.468	0.468	0.058
PMA	none	none	none	none
Cyt-D	none	none	none	none

Data representing lowest effective doses (LOED) in µM of all compounds tested compared with Cellomics criteria or one-way ANOVA. Each dose was done in quadruplicate, with and without cytochalasin B. "none" represents no change from basal levels/not statistically significant at any dose.

The relative potency of all compounds tested (based on Cellomics' hit criteria) for both mononucleate and binucleate cells were the same:

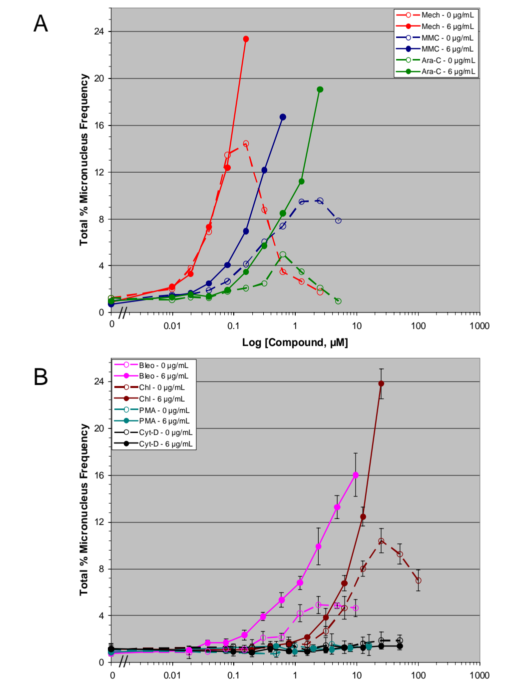
Mech>>MMC>>Ara-C>>Bleo>>>Chl

Neither PMA nor Cyt-D showed any effect in mononucleate or binucleate cells. However, PMA did show signs of toxicity (through decreases in the targeted cell count and increases in % cytotoxicity) at concentrations at and above 31.25 µM for mononucleate, and above 7.8 µM for binucleate (data not shown).

Figures 5A and 5B represent the relative potency of the dose-responses for these compounds with no Cyt-B (0 µg/mL) or 6 µg/mL Cyt-B. Figure 5A consists of the three compounds with the highest relative potency (Mech, MMC, and Ara-C). Figure 5B consists of the two weaker compounds, Bleo and Chl, as well as the two compounds that did not show any change in micronucleus frequency (PMA and Cyt-D). Doses were eliminated on the 6 µg/mL graph due to early toxicity (if the values for CBPI or ratio of multinucleate to mononucleate cells fell below our set range), or late toxicity (loss of targeted cells below 500 or increases in cytotoxicity above 50%). Doses for the 0 µg/mL could only be eliminated due to late toxicity characteristics, since early toxicity due to cell-cycle delay could not be assessed in mononucleate cells.

Overall comparison confirms the relative potency of the compounds with both the mononucleate and multinucleate cells. Each compound peaks at a similar concentration, yet the binucleate cells are at a higher micronucleus frequency, likely due to the sensitivity of selection for targeted cells. Decreases in the micronucleus frequency for the mononucleate cells are evident at higher doses for all compounds tested and are most likely due to some early stage of toxicity. The amount of time to run a plate on the instrument is faster for the mononucleate assay; however, sensitivity might be decreased, thus skewing the targeted population and making it not possible to determine if a micronucleus formation occurred due to the compound tested.

Figure 5. Genotoxicity Assessment using Mononucleate vs. Binucleate MN Assays



Dose responsiveness of micronucleus frequency in all tested compounds without Cyt-B or with 6 µg/mL Cyt-B. Each dose was done in quadruplicate, and error bars represent standard deviation. (A) Mech, MMC, and Ara-C. (B) Bleo, Chl, PMA, and Cyt-D.

Conclusions

Overall:

- Cyt-B was not found to be genotoxic at the standard concentrations and incubation times
- Cellular dye used in the Cellomics Micronucleus Reagent Kit was not found to be genotoxic/cytotoxic at the suggested concentration and incubation time
- Both mononucleate and binucleate micronucleus assays had similar results for genotoxicity of the compounds tested, as well as their relative potency
- Differences in the LOED and micronucleus frequency between assays might be due to the increased sensitivity of using Cyt-B to confirm micronucleus formation from a genotoxic agent rather than other physiological factors

The Cellomics Micronucleus BioApplication is a functional assay that can measure micronucleus frequency in both mononucleate and multinucleate populations, as well as early/late cytotoxic effects. Used with the Cellomics Micronucleus Reagent Kit and HCS Reader, it reduces subjectivity and the overall time to quantitatively analyze the images, providing an easy way to score multiple wells with various agents.

References

- Fenech M. 1997. *Mutation Res,* 392:11-8.
- Fenech M., and A. Morley. 1985. *Mutation Res.* 147:29-36.
- Fenech M., and A. Morley. 1985. *Cytobios* 43:233-46.
- Hayashi M., T. Sofuni, and M. Ishidate. 1984. *Mutation Res.* 141:165-9.
- Kirsch-Volders M., Sofuni T., Aardema M., Albertini S., Eastmond D., Fenech M., Ishidate M. Jr., Kirchner S., Lorge E., Morita T., Norppa H., Surralles J., Canhauwaert A., and A. Wakata. 2003. *Mutation Res.* 540:153-163.
- Maier P., and W. Schmid. 1976. *Mutation Res.* 40:325-38.
- OECD Guideline for the Testing of Chemicals – Draft Proposal for a New Guideline 487: In Vitro Micronucleus Test. 2004. First version:1-13.