Toxic concentrations of exogenously supplied methylglyoxal in hybridoma cell culture

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Abstract

Concentrations at which methylglyoxal, a by-product of cellular metabolism, can be toxic to hybridoma cell cultures were determined using exogenously supplied doses. Trypan blue cell counts of 6-well cultures incubated for 24 h with various methylglyoxal concentrations revealed inhibition of cell growth at 300 μ M and higher, with a median inhibitory concentration of 490 ± 20 μ M. The primary mode of death was apoptosis, as assessed by chromatin condensation, and the effects of methylglyoxal were observed to be complete by approximately eight hours. Yet, the impact of methylglyoxal was a function of the rate of dosing; stepwise addition of MG during the first 6 h of incubation inhibited growth but caused much less cell death than a comparable bolus dose. Inhibition of cellular metabolism by MG was found to coincide with inhibition of cell growth, with a comparable median inhibitory concentration of 360 ± 20 μ M. The effects on viable cell density and metabolism were both linear at doses approaching zero, with lowest observable effect levels of 54 and 77 μ M, respectively. These results provide quantitative estimates for concentrations of methylglyoxal that may be inhibitory to biopharmaceutical-producing cell lines.

Abbreviations: AOEB – acridine orange/ethidium bromide; CHO – Chinese Hamster ovary; LOEL – lowest observable effect level; HSD – honest significant difference; IC₅₀ – median inhibitory concentration; IMDM – Iscove's modified Dulbecco's Medium; MG – methylglyoxal

Introduction

Growth inhibition and cell death of mammalian cells used to produce biopharmaceuticals limits the efficiency of such production processes. There exists a clear need to increase biotechnology process efficiency to address a disparity between production demand and industry's limited current capacity (Walsh 2000). In a typical batch/fed-batch

culture, hybridoma cells begin exhibiting moderate to severe growth inhibition and cell death as early as 48–72 h post inoculation. Among the many suggested causes is the buildup of toxic metabolites. Lactate, ammonium, and alanine have been shown to accumulate to high levels in batch cultures (Zhou et al. 1997). Lactate and ammonia, in particular, have been examined on many occasions, and are thought to exert significant detrimental

effects on growth (Miller et al. 1988; Hassell et al. 1991; Duval et al. 1992; Omasa et al. 1992; Ozturk et al. 1992; Kromenaker and Srienc 1994; Ludemann et al. 1994; Newland et al. 1994; Lao and Toth 1997; Mastrangelo et al. 2000; Cruz et al. 2000; Patel et al. 2000). Aside from these, however, few other metabolites have been examined.

Chaplen et al. have proposed methylglyoxal ($C_3H_4O_2$, abbreviated MG) as potentially harmful to cell lines used in industry (Chaplen et al. 1996). The compound has been studied extensively in the context of diseases such as diabetes and cancer, and excellent reviews are available regarding its toxicity and harmful effects in many different settings (Kang et al. 1996; Chaplen 1998; Kalapos 1999). Briefly, the compound is thought to form chiefly as a byproduct of glycolytic metabolism through the non-enzymatic decomposition of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The major pathway for detoxification is reported to be the glyoxalase pathway, which consists of two enzymes, glyoxalases I and II, which convert the compound to D-lactate in consecutive reactions (Vander Jagt and Hunsaker 2003). Prior to entry into this pathway, however, free methylglyoxal must react with glutathione, an important antioxidant in cellular processes.

Knowledge of the compound's metabolism and reactivity has led to several theories regarding its toxic mechanism in disease pathology. Based on its reactivity with arginine, lysine, and cysteine residues, MG has demonstrated propensity to induce permanent modifications within proteins (Lo et al. 1994). Such modifications can result in loss of cellular function, and formation of advanced glycation end-products have been linked to various diabetic complications (Singh et al. 2001). Others have suggested MG is indirectly toxic to cells, inducing oxidative stress in cells through reaction with glutathione (Baynes and Thorpe 1999). Several *in vitro* toxicity studies of the compound have been conducted in attempt to clarify toxic levels and mechanisms. Exogenous methylglyoxal was shown to inhibit growth and cause cell death in human leukemia (HL60) cells at median inhibitory concentrations of 238 and 363 μM , respectively (Ayoub et al. 1993). Further studies on this cell line showed that the compound inhibited growth in the G1 phase, induced death through apoptosis, and demonstrated

selective toxicity towards rapidly proliferating cells (Kang et al. 1996). The ability for MG to induce apoptosis is further supported by work conducted on a fibrosarcoma cell line which demonstrated that TNF, acting through protein kinase A, can limit protection against methylglyoxal stress by decreasing glyoxalase I activity by phosphorylation (Van Herreweghe et al. 2002).

The metabolic characteristics of production cell lines distinguish methylglyoxal as being a potentially relevant toxin. Increased cellular metabolism is a defining characteristic of transformed cell lines (Plas and Thompson 2002). Since methylglyoxal production is closely linked to glycolytic metabolism, transformed cell lines could be producing consistently higher levels of MG than their physiological counterparts. Recently, total concentrations of MG (free plus that bound to biological ligands) have been detected as high as 310 μM in Chinese Hamster Ovary (CHO) cells (Chaplen et al. 1998). Glycation reactions, of which methylglyoxal is a key participant, have also demonstrated the potential to deactivate enzymes, a trait which could have significant impact on metabolically driven production cell lines (Yan and Harding 1999). Batch cultures are routinely cultured with high concentrations of glucose. Increased concentrations of glucose have been shown to increase methylglyoxal production in red blood cells (Thornalley 1988) and high levels of methylglyoxal have been linked to hyperglycemic conditions among diabetics (Vander Jagt and Hunsaker 2003). Production cell lines also share many similarities with cancer cell lines. Methylglyoxal has been tested as a chemotherapeutic, and malignant and apoptosis-resistant lines have been identified as expressing increased levels of glyoxalase I (Thornalley 1995). Presumably, the robust nature of these cells is due in part to their resistance to toxic effects of methylglyoxal.

The purpose of this study was to quantify concentrations at which methylglyoxal can be inhibitory to a MAb-producing hybridoma cell line. Specifically, experiments were conducted to determine the IC_{50} for inhibition of cell growth, the mode of death induced, the rapidity of effects, the effect of dosing rate, and whether metabolism is affected at sublethal doses. Previous measurements of MG and a simple model are discussed to place the data in perspective.

Materials and methods

Cell culture

Non-adherent, murine hybridoma cells (ATCC CRL1606) that secrete an immunoglobulin, (IgG) against human fibronectin were used for all experiments. During routine maintenance, cells were subcultured daily at 2.0×10^5 viable cell ml^{-1} and maintained in T-flasks (Corning Inc, NY, USA) placed in an incubator (Model 3100 series, Forma Scientific, Marietta, OH, USA) controlled at 37 °C, 95% humidity, and 10% CO_2 . Cell cultures were determined to be mycoplasma-free via a DNA fluorochrome assay conducted by Bionique Testing Laboratories (Saranac Lake, NY, USA). The serum- and hydrolystate-free medium used for maintenance and all experiments except the sublethal metabolic activity screens consisted of Iscove's modified Dulbecco's Medium (IMDM, without glutamine, Mediatech, Herndon, VA, USA) supplemented with 4.0 mM glutamine (Mediatech) and 10 mg l^{-1} insulin, 5 mg l^{-1} holotransferrin, 7.5 mg l^{-1} protease-free bovine serum albumin, 2.44 $\mu\text{l l}^{-1}$ 2-aminoethanol, 3.5 $\mu\text{l l}^{-1}$ 2-mercaptoethanol, and 10 U ml^{-1} penicillin-10 $\mu\text{g ml}^{-1}$ streptomycin (all from Sigma, St. Louis, MO, USA). Medium for sublethal metabolic activity screens consisted of bicarbonate-free/glucose-free RPMI 1640 medium (Sigma), supplemented with 10 mM glucose, 10 mg l^{-1} insulin, 5 mg l^{-1} holotransferrin, 10 U ml^{-1} penicillin-10 $\mu\text{g ml}^{-1}$ streptomycin, and 2000 mg l^{-1} sodium bicarbonate (all from Sigma).

Test chemicals and working stock solutions

Methylglyoxal solution (100 ml, Sigma) was purchased as an aqueous solution containing 42.09 wt% MG, with 7.04 wt% pyruvate as an impurity. Given a density of 1.178 g ml^{-1} , these specifications correspond to a solution containing 6881 mM MG and 942 mM pyruvate. A working stock solution containing 275.2 mM MG and 37.7 mM pyruvate was prepared by dilution of 4.0 ml of supply stock solution into 96.0 ml of autoclaved tissue culture grade water. Working stock solutions containing 37.7 mM pyruvate, 1000 mM sodium lactate, or 1000 mM sodium chloride were prepared by dissolving 0.415 g so-

dium pyruvate (Mediatech), 11.21 g of sodium lactate (Sigma), or 5.84 g sodium chloride (Sigma), respectively, into 100.0 ml of tissue culture grade water. Working stock solutions for lactate, pyruvate, and sodium chloride were sterile-filtered; all stocks were stored at 4 °C.

Lactate assay reagents A and B and resazurin reduction assay reagent for the 96-well sublethal metabolic activity assay were prepared exactly as reported (Yang and Balcarcel 2004).

Screening for inhibition of cell growth

The effects of MG and lactate on the viable cell density over a 24-h period were determined using 2.0 ml cultures placed within wells of 6-well plates (Nuclon delta multidish, VWR International, Suwanee, GA, USA). Six individual screens were conducted on separate days (with different inocula): three for MG and three for lactate. During a single screen, control cultures and those with either six MG concentrations (300, 400, 600, 800, 1000, and 1200 μM) or five lactate concentrations (20, 40, 60, 80, and 100 mM) were tested in duplicate wells. Screens were initiated by the mixing of 1.0 ml of a hybridoma cell culture, of density 1.0×10^6 cell ml^{-1} and viability of 96–98%, with an equal volume of fresh IMDM supplemented with a certain concentration of a compound ('test media'). The culture used was prepared by centrifuging an inoculum culture at 200 g for 10 min and resuspending in fresh medium. Test media were prepared using working stock solutions for MG, lactate, and pyruvate, as listed in Tables 1 and 2. Additional solvent and osmolality controls were included during testing of lactate. At the end of the 24-h incubation period for each experiment, viable cell density was measured by trypan blue dye exclusion counts via microscopy (Model BX60, Olympus, USA) with a hemacytometer (Double Neubauer Counting Chamber Set, VWR International, Suwanee, GA, USA).

Assessment of mode of death

The extents of apoptosis and necrosis resulting from exposures of hybridomas to MG or lactate were determined by fluorescence microscopy using an acridine orange, ethidium bromide (AOEB) dye

Table 1. Methylglyoxal test media^a for screening for inhibition of cell growth.

Concentration ^b	Volume (μ l)		
	IMDM	275.2 mM MG	37.7 mM Pyruvate ^c
Control	10000	0.0	88.0
300 μ M	10000	22.0	66.0
400 μ M	10000	29.3	58.7
600 μ M	10000	44.0	44.0
800 μ M	10000	58.7	29.3
1000 μ M	10000	73.3	14.7
1200 μ M	10000	88.0	0.0

^aCalculated osmolality of each medium was 293 $\pm$ 1. pH was 7.2 for all test media.

^bConcentrations shown are those achieved upon 1:1 dilution.

^cPyruvate is present at 0.164 mM in MG test media.

mixture (Sigma) (Mercille and Massie 1994). Following 24-h incubations with either 600 μ M MG or 80 mM lactate, cells were scored into one of four groups during fluorescence microscopy by visual assessment of dye exclusion and chromatin condensation: (1) viable, (2) early apoptotic, (3) necrotic, and (4) late apoptotic. A T-25 cm² flask containing 7.5 ml of culture was prepared for each compound for each test, and the test was accomplished on three independent occasions. Each flask received 3.75 ml of culture and 3.75 ml of test media, yielding final concentrations of either 600 μ M MG or 80 mM lactate. Cultures used were prepared by centrifugation at 200 $\times$ g for 10 min of a parent culture of density near 1.0×10^6 cell ml⁻¹

and viability 96-98%, followed by resuspension in fresh IMDM medium at 6×10^5 viable cell ml⁻¹. Test media were prepared as done for screening of inhibition of cell growth experiments.

Measurement of time required to observe toxic effects of exogenous doses of MG

Using the same method described for the screening experiments, MG concentrations of 600 and 800 μ M were re-tested during incubations lasting only 2.7, 5.3, or 8.0 h. For a single test, three 6-well plates were prepared, each with two control cultures, two cultures with 600 μ M MG, and two cultures with 800 μ M MG. At the end of each of the specified incubation times, two control well cultures and two well cultures for each concentration tested were counted using Trypan blue exclusion. Three independent tests were conducted on separate days.

Comparison of bolus and gradual exposure to MG

Tests of inhibition of cell growth over 24 h for cultures exposed to 600 μ M MG were repeated to explore the effect of exposure method. In each of three tests, three T-25 cm² flasks were prepared with 7.5 ml of hybridoma cell culture of density 5×10^5 viable cell ml⁻¹ and viability 96-98%. A bolus dose of 66.0 μ l of a 68.8 mM MG solution (prepared by 4-fold dilution of the working MG

Table 2. Lactate and NaCl test media^a for screening for inhibition of cell growth.

Concentration ^b	Volume (μ l)					Percent solvent	Osmolality (mOsm/kg)
	IMDM	1 M Lactic acid	4 M NaOH	1 M NaCl	Water		
Control 1	8000	0	0	0	421	2.6	287
20 mM NaCl	8000	0	0	337	84	2.6	327
20 mM Lac	8000	337	84	0	0	2.6	327
40 mM NaCl	8000	0	0	711	178	5.6	359
40 mM Lac	8000	711	178	0	0	5.6	359
60 mM NaCl	8000	0	0	1130	282	8.8	392
60 mM Lac	8000	1130	282	0	0	8.8	392
80 mM NaCl	8000	0	0	1600	400	12.5	425
80 mM Lac	8000	1600	400	0	0	12.5	425
Control 2	8000	0	0	0	2666	16.7	257
100 mM NaCl	8000	0	0	2133	533	16.7	457
100 mM Lac	8000	2133	533	0	0	16.7	457

^aOsmolality for each test media was estimated by calculation. pH was 7.2 for all test media.

^bConcentrations shown are those achieved upon 1:1 dilution.

stock with tissue culture grade water) was given to one flask at time zero. The other two flasks received 11.0 μl of either 68.8 mM MG solution or pure water at the beginning of each of the first 6 h. Viable cell density of each flask was determined at the end of 24 h by cell counts using Trypan blue.

96-well plate assay for sublethal metabolic activity

The possibility of MG causing adverse affects on metabolism at doses lower than those causing growth inhibition and cell death was investigated using a 96-well plate assay for sublethal metabolic activity (Yang and Balcarcel 2004). In this implementation, changes in average lactate production rate relative to control cultures during 10-h exposures were obtained along with relative resazurin reduction rates in the final 2 h of the screen. Concentrations tested were 0, 54, 77, 109, 155, 220, 312, 443, 629, 893, 1268, and 1800 μM (prepared by serial dilution by factor 1.42).

The 96-well plate was prepared by adding 50 μl of MG-free test medium to each well using an 8-channel, 5–120 μl electronic pipettor (e-line, Biohit Inc, KSE Scientific, Durham, NC, USA). Next, 119 μl of a MG working solution of concentration 5112 μM (prepared by mixing 400.0 μl of the working MG stock into 21.13 ml of test medium in a 50-ml centrifuge tube) was added to wells of the 12th column of the 96-well plate and mixed three times by pipettor action. Then, 119 μl of solution were transferred to the 11th column and mixed. Transfers and mixings were repeated down the plate until column two, at which point the 119 μl were discarded, leaving wells of column 1 as MG-free controls. Next, 50 μl of cell-free test medium was added to rows A, B, and C and 50 μl of 1.5×10^6 viable cell ml^{-1} culture in test medium was added to rows D-H, using a 12-channel, 5–120 μl electronic pipettor (e-line, Biohit Inc.). Like for other experiments, the cell culture was obtained from a parent flask that was counted using Trypan Blue, centrifuged, and resuspended at the desired cell density. The well plate was incubated at 37 °C, 95% humidity, and 10% CO_2 for 8 h. Next, 10 μl of resazurin reagent solution was added to each well using the 12-channel electronic pipettor working by row. Then the plate was incubated for 2 more hours. At the 10 h mark, the

fluorescence of each well of the plate was read using wavelengths of 530/590 on a FL600 plate reader (Bio-Tek® Instruments, Winooski, Vermont, USA). Then, the cultures were centrifuged at $200 \times g$ for 10 min within the same plate (using the special attachment on the centrifuge) and 20 μl volumes of supernatant were sampled with a 12-channel pipettor working by row and transferred directly into a 96-well plate prepared with 170 μl of lactate reagent A in each well. Initial absorbencies were read at a wavelength of 340 nm using μ -Quant spectrophotometer (Bio-Tek® Instruments). 10 μl of lactate reagent B were added to each well by row using the 12-channel electronic pipettor. This assay plate was incubated for 2 h at room temperature and final absorbencies read at 340 nm. This screen was conducted in duplicate. Results shown are the average of the two screens. Data was reduced as reported (Yang and Balcarcel 2004).

Data analysis

Data from replicates were combined and analyzed as a conglomerate. Dunnett's method was used to compare means of test concentrations to means of controls. Tukey–Kramer honest significant difference (HSD) was used to compare pairs of means within a data set. Analysis was conducted using Microsoft Excel and JMPIN software version 4.0.2 (SAS Institute, Inc) and statistical significance was determined at the 95% confidence level.

Data for inhibition of cell growth from 24-h screens and lactate production rate and resazurin reduction from 10-h sublethal metabolic activity screens were fitted to the following Sigmoidal equations using Matlab Version 6.5 Release 13 (The Mathworks, Inc):

$$f(x) = 100 / (1 + \exp(-a(x - b))) + c \quad (1)$$

$$f(x) = 100 / (1 + \exp(-a(x - b))) \quad (2)$$

In these equations, $f(x)$ represents the data being fitted, x the concentration of MG, and a , b , and c empirical parameters. Equation (2) was used for the viable cell density data. Equation (1) was used for sublethal metabolic activity data for purposes of graphing the results. Equation (2) was used to determine IC_{50} values from data that was re-scaled

from a 0–100% basis to minimum value to 100% basis. In the second equation, parameter b represents the IC_{50} and the errors quoted on it are the 95% confidence interval from the nonlinear regression.

To ascertain lowest observed effect levels (LOELs) from sublethal metabolic activity data sets, 10 fluorescence or absorbance values for each test concentration were compared to mean values of the control ($n = 10$) using a 1-tailed t -test (with degrees of freedom equal to 18). The values used from each screen were normalized to the mean values of their controls to block any variations due to factors besides concentration of MG.

Model for total methylglyoxal concentration in batch/fed-batch culture

Mammalian cells produce MG as a by-product of glycolysis, as well as several other pathways (Kang et al. 1996; Chaplen 1998; Kalapos 1999). In one study conducted with red blood cells, the amount of MG produced per glucose consumed ranged from 0.2 to 0.8% on a mole basis (Thornalley 1988). This amount was estimated from the production of D-lactate. The production rate could actually be higher if one takes into account other possible fates of MG. For the purposes of this work, we simply desired to estimate an approximate level to which MG might be accumulating during a batch or fed-batch culture. To this end, we simulated a fedbatch culture by specifying the growth rate, death rate, and specific glucose uptake rate (based on previously obtained data from our cell line) typical of our cell line. Then we simply modeled production of MG as a fraction of glucose uptake, varying this fraction as a design variable. The output of the model is the number of viable cells, the number of total cells, and the total concentration of MG in the culture. The concentration of MG is for the entire volume of the cell culture, as opposed to cellular volume, given that MG is quoted to be freely permeable to cellular membranes (Chaplen et al. 1998; Thornalley 1988).

The following equations were used to simulate the growth, death, and glucose uptake rate of CRL1606 hybridomas during 96 h of a fed-batch, and provide estimates for total MG concentration in the cell culture. The ordinary differential equa-

tions were numerically integrated using Matlab (The Mathworks) and results were plotted using Excel (Microsoft).

Growth rate(hr^{-1}):

$$\mu(t) = 0.1168e^{(-0.0262t)} - 0.0940e^{(-0.0661t)} \quad (3)$$

Death rate (hr^{-1}):

$$k_d(t) = 8.100 \times 10^{-4} - 8.268 \times 10^{-5}t + 1.990 \times 10^{-7}t^2 + 1.033 \times 10^{-7}t^3 - 7.869 \times 10^{-10}t^4 \quad (4)$$

Glucose uptake rate ($\mu\text{mol viable-cell}^{-1}\text{hr}^{-1}$):

$$q_{\text{glc}}(\mu) = 6.5 \times 10^{-6}\mu + 1.0 \times 10^{-7} \quad (5)$$

Fraction of glycolysis contributing to MG production:

$$f_{\text{MG}} \in (0.002, 0.004, 0.006, 0.008) \quad (6)$$

$$\begin{aligned} \text{Viable cell density (viable cell l}^{-1}\text{): } \frac{dn_v}{dt} \\ = (\mu - k_d)n_v \end{aligned} \quad (7)$$

$$\text{Total cell density (cell l}^{-1}\text{): } \frac{dn_T}{dt} = \mu n_v \quad (8)$$

Total MG concentration(μM):

$$\frac{dC_{\text{MGtot}}}{dt} = f_{\text{MG}}q_{\text{glc}}n_v$$

Results

Significant inhibition of cell growth was evident for methylglyoxal concentrations of 300 μM or higher and lactate concentrations of 40 mM and higher during 24-h incubations (Figure 1a, b). Test media during experiments with MG had similar osmolality; thus toxicity was not as a result of osmolality differences. In contrast, tests conducted with NaCl alongside those of lactate revealed that much of the effect of lactate is due to increased osmolality rather than innate toxicity, as reported

Figure 1. Viable cell densities (black bars for MG and lactate, gray bars for sodium chloride, and stripped bars for controls) and non-viable cell densities (white bars), as determined by Trypan Blue cell counts, after 24 h exposure to various doses of methylglyoxal (a) and sodium lactate or sodium chloride (b). All cultures were seeded with $5.0 \times 10^5 \text{ cell ml}^{-1}$. Values are the mean $\pm$ standard deviation of six wells measured during three independent replicates, with the exception of the control for methylglyoxal data which is based on 18 wells. Asterisks indicate statistical difference from control cultures (control 1 for lactate and sodium chloride experiments). Daggers placed between pairs of data indicate statistical difference according to the Tukey Kramer HSD test.

previously (Ozturk et al. 1992; Lao and Toth 1997; Cruz et al. 2000). The use of an additional control with maximum amount of solvent (control 2 with 16.7% vol $\text{H}_2\text{O}/\text{vol}$) further indicates that dilution of medium is not the cause for inhibition of cell growth. The nonviable cell density data further reveal that effects of methylglyoxal are weighted more towards causing cell death than those of lactate/NaCl/osmolality. For similar reductions in viable cell density (i.e. 600 μM MG vs. 80 mM lactate), cultures exposed to MG exhibited larger nonviable cell densities. The amount of death due

to MG may even be underestimated here, considering that MG was observed to cause appreciable cell lysis at higher doses (i.e. final total cell density was less than initial one of $5.0 \times 10^5 \text{ cell/ml}$). Qualitatively, considerable cell debris was noted during trypan blue cell counts for MG tests but not those for lactate or NaCl tests. For this reason, we quantified toxic effects using viable cell density rather than total cell density. According to Sigmoidal curve fits of viable cell density vs. dose using Eq. (2), methylglyoxal had a median inhibitory concentration of $490 \pm 20 \mu\text{M}$ as compared to $77 \pm 4 \text{ mM}$ for lactate.

The extent of apoptosis caused by MG and lactate was determined for concentrations which had elicited approximately 50% inhibition of cell growth during the 6-well plate experiments, namely 600 μM MG and 80 mM lactate (Figure 2). The AOEB viabilities for MG-or lactate-treated cultures were $44 \pm 29\%$ and $88 \pm 1\%$, respectively, indicating again that MG caused higher levels of cell death than did lactate for similar degrees of inhibition of cell growth. The AOEB data further indicated that $81 \pm 7\%$ of cells which died in MG-treated cultures exhibited chromatin condensation, distinct from $37 \pm 11\%$ for lactate-treated cultures. The variability in the data appears to be the result of variance in the

Figure 2. Fractions of viable (white), early apoptotic (black), necrotic (diagonal lines), and late apoptotic (grid) cells following 24 h exposures to methylglyoxal (600 μM) and lactate (80 mM). Cells were scored into one of four groups during fluorescence microscopy by visual assessment of dye exclusion and chromatin condensation. Values shown are the mean $\pm$ standard deviation for three cultures tested.

number of cells that died, not the mode in which they died (e.g. noise on apoptosis ratio of 7% is less than that of 24% for viability).

In order to determine the rate at which methylglyoxal affects hybridoma cultures, viability was measured during the first 8 h of MG exposure (Table 3). Significant reductions in viable density were seen at 5.3 and 8.0 h, but not 2.7 h, for both doses tested. Comparison of data from 5.3 and 8.0 h, as well as outcomes of 24-h exposures described above, indicates that the effects of MG have been manifested by 8 h of exposure. Absolute viable cell densities at 8 h for both doses of 600 and 800 μM (not shown) were in fact equal to or less than those observed at the end of 24 h exposures. Lesser densities at 8 h compared to 24 h could be due to experimental variation or could possibly indicate the growth of surviving cells.

The extent to which the rate of MG accumulation in a hybridoma culture plays a role in growth inhibition and cell death was investigated by comparing the effects between a bolus dose (as in the previously described experiments) against those of gradual dose. A 600 μM dose was applied in two different modes to the culture: as a bolus dose at time zero and as six equally sized doses at the beginning of each of the first 6 h. Then, after 24 h from the initiation of the experiment, the cultures were counted via trypan blue exclusion (Figure 3). The data reveal that the viable cell density of cultures administered a gradual dose were still affected but to a much lesser degree. Furthermore, cultures given a gradual dose retained a higher viability. The reason behind this phenomenon is not clear. One possibility is that the slower rate of addition allowed for some processing or degradation of MG, such that the peak

Table 3. Viable cell density^a during the early hours of methylglyoxal exposure.

Trial	Concentration (μM)	2.7 h	5.3 h	8.0 h
1	600	1.01 ± 0.31	0.35 ± 0.10	0.23 ± 0.03
	800	0.95 ± 0.17	0.21 ± 0.03	0.11 ± 0.04
2	600	0.95 ± 0.06	0.34 ± 0.01	0.19 ± 0.00
	800	0.99 ± 0.16	0.28 ± 0.02	0.08 ± 0.00
3	600	1.05 ± 0.26	0.43 ± 0.01	0.46 ± 0.03
	800	0.99 ± 0.16	0.17 ± 0.02	0.15 ± 0.02

^aFor each trial, data is normalized to controls and error shown is standard deviation of two wells measured.

Figure 3. Effect of dosing mode on viable (black) and non-viable (white) cell densities as well as viability (line) after 24 h exposure to methylglyoxal in T-flasks. A set of six equally sized doses given at the beginning of each of the first six hours, for a total of 600 μM , vs. a bolus dose of the same total concentration applied all at once at $t = 0$. Values are mean $\pm$ standard deviation of three separate tests. Both exposures are statistically different from the control (indicated by asterisks), as well as from each other (daggers).

concentration was less than 600 μM . In fact, the result of a bolus dose of 400 μM shown in Figure 1a is not too dissimilar to that of the gradual dose shown here. Alternately, these results could indicate a cellular adaptation, such as induction of detoxification pathways, stimulated by the initial lower doses. Whatever the explanation, the reduced incidence of cell death following a gradual dose suggests that hybridomas may perhaps be able to withstand higher doses over time than those observed to be detrimental as bolus doses.

A final experiment was conducted to explore lethal, and possibly sublethal metabolic, effects of concentrations lower than seen during 24-h screens (i.e. 300 μM) and to obtain estimates for lowest observable effect levels (LOELs). Figure 4 depicts the effect of MG exposures during duplicate, 10-h, 96-well plate sublethal metabolic activity assays. Groups of 100- μl hybridoma cultures were exposed to eleven concentrations spanning 54–1800 μM MG, and results normalized to the MG-free control group. The IC₅₀'s for inhibition of cell growth and inhibition of average lactate production rate were estimated to be 480 ± 20 and 364 ± 20 μM , respectively. The LOEL's for each effect were 54 and 77 μM , respectively. Notable is the absence of any apparent threshold in the effects observed. The slopes of each effect in the vicinity of zero dose appear to be nonzero, with effect

Figure 4. Resazurin reduction (a) and average lactate production rate (b) relative to control cultures during 10-h exposure to various concentrations of methylglyoxal. Values are mean $\pm$ standard deviation of two replicates. All data for test cultures were significantly different from their respective controls, with the exception of the average lactate production rate at 54 μM . IC_{50} 's quoted are those calculated between 100% and the minimum value observed. LOELs were statistically different from controls with p -values of 0.0002 and 0.0262, respectively.

increasing linearly as dose increases. This might suggest that detoxification of MG is not rapid enough to handle bolus increases in MG, even for low doses.

Discussion

The work provides the first quantitative estimates for exogenously supplied MG concentrations that are observably detrimental to hybridoma cell cultures. IC_{50} 's for inhibition of cell growth due to bolus exposures by MG were found to be

$490 \pm 20 \mu\text{M}$ (by trypan blue assays after 24 h) and $480 \pm 20 \mu\text{M}$ (by resazurin reduction assays after 10 h). Effects of a bolus dose appear to be complete at around 8 h (by TB assays as well as in noting the similarity of 10 vs. 24-h IC_{50} 's) and were found to be largely due to apoptosis (according to AOEB assays). The lowest observable effect level on viable cell density was measured to be 54 μM , which was the lowest concentration tested in the sublethal metabolic activity screens. At this dose, the number of viable cells in the test wells was $6 \pm 1\%$ lower than those of controls. Metabolic effects occurring in the absence of inhibition of cell growth were not observed, an impact distinct from that observed for metabolic inhibitors (Yang and Balcarcel 2004). Inhibition of lactate production, characterized by an LOEL of 77 μM and IC_{50} of $364 \pm 20 \mu\text{M}$, coincides with appreciable inhibition of cell growth.

For all practical purposes, the concentrations of MG within cells and in the extracellular medium should be similar after an initial period of equilibration since MG is freely permeable to cell membrane (Thornalley 1988). However, most MG in living systems is bound (in many cases reversibly) to biological ligands, a good portion of which are proteins (Lo et al. 1994; Chaplen et al. 1998), and MG is also reversibly hydrated to mono- and bis-hydrate forms (Rae et al. 1990). The concentrations quoted in this study should be taken to be total MG concentrations. What remains unclear in our screens is the distribution of MG between the aforementioned forms. It is also not clear how much MG could have reacted with GSH and been detoxified. The extension of results obtained from exogenously supplied, bolus doses to endogenously generated, gradual doses further warrants some caution. Only in the case of limiting reaction kinetics relative to fast convective and diffusive transport would bolus and gradual accumulations result in similar outcomes. This is not likely, however, and the 'buffering' of toxic outcome observed in this work for gradual dosing is a testament to the complexity of the underlying mechanisms determining the fate of the cells upon exposure to MG.

Concentrations of free MG and MG bound to biological ligands for unexposed CHO cells (containing MG due to endogenous production) have been determined via carefully made HPLC measurements (Chaplen et al. 1996; Chaplen

1998). Free concentrations are reported to be in the range of 0.5–2 μM , while total concentrations (free plus bound MG) were reported to be in the range of 80–100 μM , and even 310 μM in one instance. At face value, our data would indicate that the levels quoted for the CHO cells correspond to situations just within the observable range of toxicity: 80–310 μM are below the IC_{50} but above the LOEL. Of course, this comparison is between different cell lines. Further still, accumulation by endogenous production (presumably quite gradual) would presumably dampen the observable symptoms. The CHO cell cultures used to obtain the reported measurements were taken during the mid-exponential phase and were, presumably, not dying at the time. Thus, the LOEL and IC_{50} values we report may be lower due to differences between mode of accumulation and possibly differ due to cell line specificity.

To put concentrations like 54 μM and 480–490 μM into perspective, we devised the simplest of mathematical models for estimating accumulation of total MG during a simulated fed-batch hybridoma cell culture. Simulated concentrations indicate that MG could be a problem in our culture systems (Figure 5b). Starting with an initial condition of zero MG in the cell culture, we see that MG production rates of 0.4% of glycolysis and higher could be cause for concern, yielding concentrations greater than the LOEL by 48 h but not reaching the IC_{50} even after 96 h.

Conclusion

Concentrations at which MG adversely affects viable cell density of hybridoma cultures have been quantified using exogenously supplied doses. In this study, the LOEL and IC_{50} for inhibition of cell growth were 54 μM and of 480–490 μM , respectively. Sublethal metabolic effects below these doses were not observed. While literature findings for measured levels in CHO cells and a simple model indicate that these types of concentrations are feasibly achieved in cell culture, more work is needed to understand the differences in culture responses due to endogenous vs. exogenous MG, which involves a study of the kinetics of its formation, hydration, detoxification, modification of biological ligands, and transport out of the cell.

Figure 5. Simulated viable (dashed line) and total (solid line) cell density for a hybridoma fedbatch culture (a). Simulated total methylglyoxal concentrations at 48 and 96 h (b) for methylglyoxal production rates specified as 0.2% (black), 0.4% (diagonal), 0.6% (grid), and 0.8% (white) of the glucose uptake rate.

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