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A Novel Cytotoxicity Screening Assay Using a Multiwell Fluorescence Scanner[1]

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A new assay using a multiwell fluorescence scanner was developed for screening cytotoxicity to cells cultured in 96-well microtiter plates. The assay is based on binding of propidium iodide to nuclei of cells whose plasma membranes have become permeable due to cell death. Fluorescence of propidium iodide measured with a multiwell fluorescence scanner increased in proportion to the number of permeabilized cells. After ATP depletion of hepatocytes and neonatal cardiac myocytes with metabolic inhibitors ("chemical hypoxia"), and exposure of Madine Darby canine kidney cells to the toxic chemical, $HgCl_2$, propidium iodide fluorescence progressively increased. Increases of fluorescence were linearly proportional with release of lactate dehydrogenase into the culture medium. Employing this cytotoxicity screening assay, protection by various agents against lethal injury was evaluated in cultured hepatocytes during chemical hypoxia. Inhibitors of cysteine proteases (i.e., antipain, leupeptin, E-64), serine proteases (i.e., PMSF), and aspartic acid proteases (i.e., pepstatin A) did not protect against chemical hypoxia. In contrast, 1,10-phenanthroline, an inhibitor of metalloprotease, markedly protected against the onset of cell death during chemical hypoxia. Half-maximal protection after 60 min occurred at 0.5 μM. Phospholipase inhibitors, chlorpromazine (50 μM) and mepacrine (50 μM), also substantially retarded cell killing. U74006F, an inhibitor of lipid peroxidation, slowed cell killing to a lesser extent during chemical hypoxia and after oxidative stress with *t*-butyl hydroperoxide. Calciphor, a dimer of prostaglandin B_1, did not protect against cell killing during chemical hypoxia or *t*-butyl hydroperoxide toxicity. In conclusion, this high capacity cytotoxicity assay for cells cultured in 96-well microtiter plates is suitable for rapid screening of potential cytoprotective agents in a variety of cell types. © 1992 Academic Press, Inc.

Each year, thousands of new chemical compounds are synthesized by the chemical and pharmaceutical industries. Compounds showing economic promise must be screened for toxicity. Initial screening determines the dose that produces 50% killing of test animals. Such testing is expensive, consumes large numbers of animals, and has been criticized as inhumane. In response to these issues, manufacturers of pharmaceuticals, pesticides, cosmetics, and household products are evaluating methods to reduce the number of animals used in toxicity screening (Holden, 1989). Increasingly, manufacturers view methods that employ cell and tissue culture techniques as economically and scientifically preferable. In particular, the Ames test is widely employed as a preliminary assay for mutagenicity and carcinogenicity (Ames *et al.*, 1972).

A variety of biochemical and morphological tests have been employed to assess cytotoxicity of chemical substances *in vitro*. Because the plasma membrane is essential to cell function and viability, most common indicators of cell viability monitor plasma membrane integrity. Nuclear staining by membrane-impermeant dyes (e.g., trypan blue, ethidium bromide, propidium iodide), release of trapped cytoplasmic probes (e.g., fluorescein, ^{51}Cr-labeled protein) and leakage of intracellular enzymes (e.g., lactate dehydrogenase, transaminases, creatine phosphokinase) all have been accepted as appropriate indicators of irreversible cell damage (Spangberg, 1973; Bhuyan *et al.*, 1976; Story *et al.*, 1983; Green *et al.*, 1984; Cheung *et al.*, 1985; Gores *et al.*, 1988). Indeed, the plasma membrane literally ruptures at the onset of cell death. This irreversible event is followed quickly by loss of cytosolic probes and enzymes and by nuclear labeling with extracel-

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lular dyes such as propidium iodide and trypan blue (Lemasters *et al.*, 1987; Herman *et al.*, 1988; Nieminen *et al.*, 1988).

Unfortunately, many of these methods are not well suited for large-scale toxicity screening. Most are time-consuming and labor-intensive, requiring numerous manipulations such as dilutions, incubations, centrifugations, and observations with a microscope. Enzyme assays may use expensive reagents, and indicator enzymes may be inhibited by some toxicants, distorting the results. Furthermore, most assays destroy some or all of the cells whose viability is being monitored. Thus, such assays are unsuitable for repetitive, serial measurements. Therefore, a need exists for a cytotoxicity assay that is rapid, inexpensive, amenable to automation, and capable of monitoring many samples repetitively.

Propidium iodide fluorescence has been used extensively to monitor viability of single cells by fluorescence-activated cell sorting and fluorescence microscopy (Pavlik *et al.*, 1985; Lemasters *et al.*, 1987). Like trypan blue, propidium iodide binds to the nuclei of nonviable cells, but cannot enter viable cells. Binding to double-stranded nucleic acids also causes a red shift and an enhancement of propidium iodide fluorescence. In previous work, we used these spectral changes to develop a fluorometric assay for measuring cell viability in hepatocyte suspensions continuously over time (Gores *et al.*, 1988). However, cultured cells ordinarily do not grow in suspension. Thus, the aim of this study was to adapt this cytotoxicity assay to cells in monolayer culture.

MATERIALS AND METHODS

Materials. 2-Deoxy-D-glucose (grade III), pyruvate, NADH, triethanolamine, propidium iodide, $HgCl_2$, antipain, leupeptin, chlorpromazine, mepacrine, iminodiacetic acid, PMSF, E-64, pepstatin A, 1,10-phenanthroline, trypan blue, and *t*-BuOOH were purchased from Sigma Chemical Co. (St. Louis, MO); collagenase B and D, and Hepes from Boehringer-Mannheim Biochemicals (Indianapolis, IN); KCN and NaCN from Fisher Scientific (Fairlawn, NJ); sodium iodoacetate from Aldrich Chemical Co. (Milwaukee, WIS); digitonin from Behring Diagnostics (La Jolla, CA); insulin from Squibb-Novo, Inc. (Princeton, NJ); dexamethasone sodium phosphate from LyphoMed, Inc. (Rosemont, IL); and tissue culture products from GIBCO (Grand Island, NY). U74006F and calciphor were gifts of Dr. John McCall (Upjohn Co., Kalamazoo, MI) and Dr. Thomas Devlin (Hahneman University), respectively. Other reagents were of analytical grade and obtained from the usual commercial sources.

Cell isolations and culture. Hepatocytes were isolated by collagenase perfusion of livers from fed male Sprague–Dawley rats by the method of Seglen (1976), as described (Herman *et al.*, 1988). Viability of hepatocytes was routinely >90% by trypan blue exclusion. Prior to plating, hepatocytes were resuspended in Waymouth's medium MB-752/1 containing 2 mM *L*-glutamine, 27 mM $NaHCO_3$, 10% fetal calf serum, 100 nM insulin, and 10 nM dexamethasone. Myocytes were isolated from 2 to 3-day-old rat neonates by enzymatic digestion (Harary and Farley, 1963) and purified by counterflow elutriation by modification of the method of Ulrich *et al.* (1989), as described by Bond *et al.* (1991a). Purified myocytes were then suspended in Eagle's minimum essential medium containing 5% fetal calf serum and 10% horse serum. MDCK[5] cells were obtained from American Type Culture Collection (Rockville, MD). These cells were grown in 25-cm^2 cell culture flasks in Dulbecco's modified Eagle's medium with Ham's F-12 1:1 mixture containing 15 mM NaHepes buffer, 2 mM *L*-glutamine, sodium bicarbonate, and 10% fetal calf serum. For replating, cells were trypsinized and suspended in growth medium. The various cell types were plated on Falcon flat-bottom 96-well microtiter plates (Becton–Dickinson, Lincoln Park, NJ) at various densities in 200 μl of culture medium and incubated at 37°C with humidified 95% air and 5% CO_2.

Fluorescence spectra of propidium iodide. Fluorescence excitation and emission spectra in cell suspensions were collected with a Perkin–Elmer Model 850-40 fluorescence spectrophotometer equipped with a magnetic stirrer and warmed with recirculating water at 37°C.

Fluorescence measurements from microtiter plates. Cytotoxicity assays were performed in a Titertek Fluoroskan II fluorescence scanner (Flow Laboratories, McLean, VA) using 544-nm (18-nm band pass) excitation and 605-nm (long pass) emission filters, a Pandex fluorescence scanner (Baxter Health Care, Mundelein, IL) using 545-nm excitation and 575-nm emission interference filters (bandpass information not available), and a CytoFluor 2300 scanner (Millipore, Bedford, MA) using 560-nm (40-nm band pass) excitation and 645-nm (50-nm band pass) emission filters.

Cytotoxicity assay. Cells cultured on microtiter plates were washed once with KRH. To each well, 100 μl of KRH containing propidium iodide and potential cytoprotective agents to be tested were then added. After 10 min incubation at 37°C, initial fluorescence from each well was measured. After the total of 30 min incubation, the toxic reagents of interest were added. Subsequently, fluorescence was measured at frequent intervals, typically every 15 min. Between measurements, microtiter plates were placed in a 37°C incubator. At the end of the experiment, digitonin (375 μM) was added to each well to permeabilize all cells and label all nuclei with propidium iodide. Fluorescence was measured again to obtain a value corresponding to 100% cell death. Percentage viability (V) was calculated as $V = 100(X-A)/(B-A)$, where A is initial fluorescence, B is fluorescence after addition of digitonin, and X is fluorescence at any given time (Gores *et al.*, 1988). In preliminary experiments, a wide concentration range of potential protective agents was screened. In most instances, the minimum concentration providing maximum protection is illustrated in the results.

Lactate dehydrogenase. Lactate dehydrogenase (LDH) in the incubation medium was measured as described (Bergmeyer and Bernt, 1974). LDH released into the medium following exposure to 375 μM digitonin was taken as 100% of total activity.

Statistical analysis. Data plotted are means from fluorescence measurements of four or more wells unless otherwise indicated. The standard errors of the means are shown as error bars when not smaller than symbol size. Difference between means were assessed by the Student *t* test, using $p < 0.005$ as the criteria of significance.

RESULTS

Fluorescence spectra of propidium iodide. To determine optimal excitation and emission wavelengths for the cytotoxicity assay, we recorded excitation and emission spectra for propidium iodide in the presence and absence of digitonin-permeabilized hepatocytes using a dual monochromator spectrofluorometer. In the absence of cells, propidium iodide had excitation and emission maxima near 500 and 625 nm, respectively, (Fig. 1, no cells). When digitonin-permeabilized hepatocytes (10,000–100,000/ml) were added, an increase of fluorescence and a red shift of the excitation spectra of propidium iodide occurred (Fig. 1). A similar fluorescence enhancement occurred for the emission spectrum with a slight blue shift. In the absence of digitonin, the changes to propidium iodide fluorescence caused by addition of viable

[5] Abbreviations used are KRH, Krebs–Ringer–Hepes; MDCK, Madine Darby canine kidney; MTT, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; *t*-BuOOH, *t*-butyl hydroperoxide.

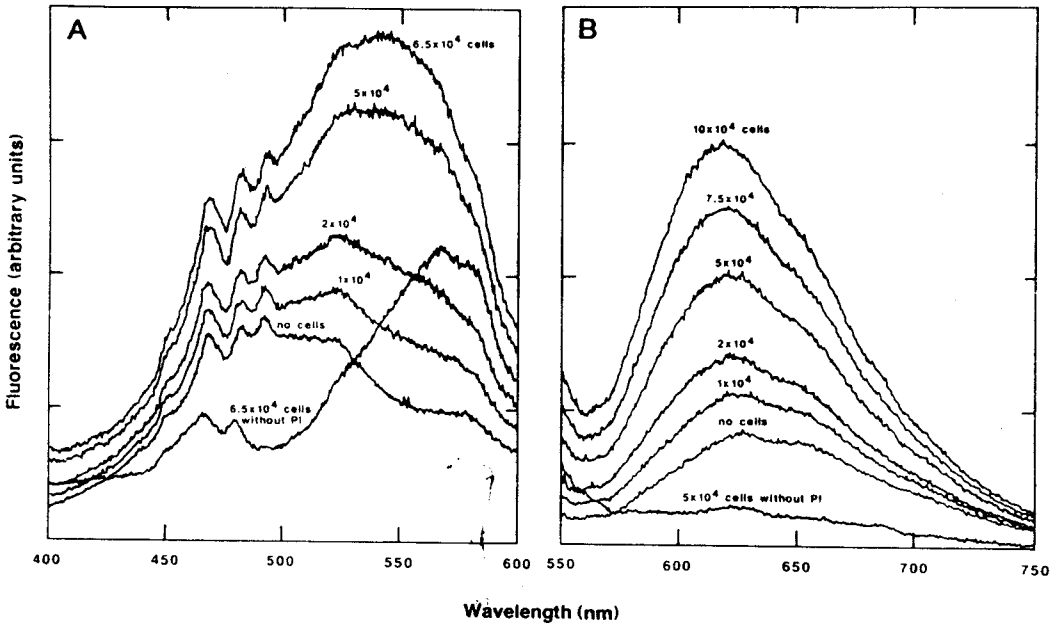

FIG. 1. Fluorescence excitation and emission spectra of propidium iodide in the presence of digitonin-permeabilized hepatocytes. Fluorescence excitation spectra at 640 nm emission (A) and emission spectra at 515 nm excitation (B) were recorded from hepatocytes (10,000–100,000) suspended in Krebs-Henseleit-Hepes buffer containing 10 mM glucose, 1.0 μM propidium iodide, and 500 μM digitonin. Minor peaks are lamp artifacts. Propidium iodide (PI) was omitted as indicated.

hepatocytes were essentially absent. From these measurements, we plotted a difference spectrum representing changes of fluorescence attributable to interaction of propidium iodide with permeabilized cells (Fig. 2). The largest change occurred at excitation and emission wavelengths of 540 and 610 nm, respectively.

Relation of cell plating density and propidium iodide concentration to fluorescence intensity of digitonin-permeabilized cultured hepatocytes. To determine optimal conditions for measurement of propidium iodide fluorescence from microtiter wells, dye concentration and cell number per well were varied simultaneously. Digitonin was added to permeabilize all hepatocytes. Digitonin binds cholesterol and permeabilizes plasma membranes (Fiskum *et al.*, 1980), rapidly permitting propidium iodide to enter cells and bind to chromatin (Gores *et al.*, 1988). With each of the three fluorescence scanners, propidium iodide fluorescence increased linearly with cell number up to 75,000 cells/well, as illustrated in Fig. 3 for the Pandex scanner. Fluorescence over baseline increased as a function of propidium iodide concentration. However, dye concentration higher than 50 μM did not increase fluorescence further (Fig. 3). Therefore, we selected a propidium iodide concentration of 50 μM for subsequent experiments with the Pandex scanner. Due to differences in filter settings between the three instruments, dye concentration was optimized to each scanner individually. Based on these results, we used a propidium iodide concentration of 5 μM for the Fluoroskan II and 30 μM for the CytoFluor 2300. Cell plating density varied between 35,000 and 50,000 cells per well, as indicated in each experiment.

Correlation between propidium iodide fluorescence and lactate dehydrogenase release. In a previous study in cell suspensions, we showed that release of LDH was linearly related to trypan blue and propidium iodide labeling of nuclei and to total fluorescence of propidium iodide (Gores *et al.*, 1988). To extend this finding to plated cells, we measured LDH release in relation to propidium iodide fluorescence of 1-day cultured hepatocytes in 96-well microtiter plates. Cultured hepatocytes were exposed to 2.5 mM KCN and 0.5 mM iodoacetic acid, and propidium iodide fluorescence was measured using a fluorescence scanner. Simultaneously, aliquots of supernatant were removed and analyzed for LDH activity. The results showed that propidium iodide fluorescence was linearly proportional to LDH release into the extracellular buffer (Fig. 4). However, increases of propidium iodide fluorescence preceded slightly the corresponding increases of LDH release. This discordance probably reflects the time required for LDH to diffuse into the bulk unstirred medium from plated cells losing viability.

Validation of cytotoxicity assay with different cell types. To validate the propidium iodide cytotoxicity assay with different cell types, we exposed hepatocytes, neonatal cardiac myocytes, and MDCK cells to toxic chemicals and monitored propidium iodide fluorescence. One-day cultured

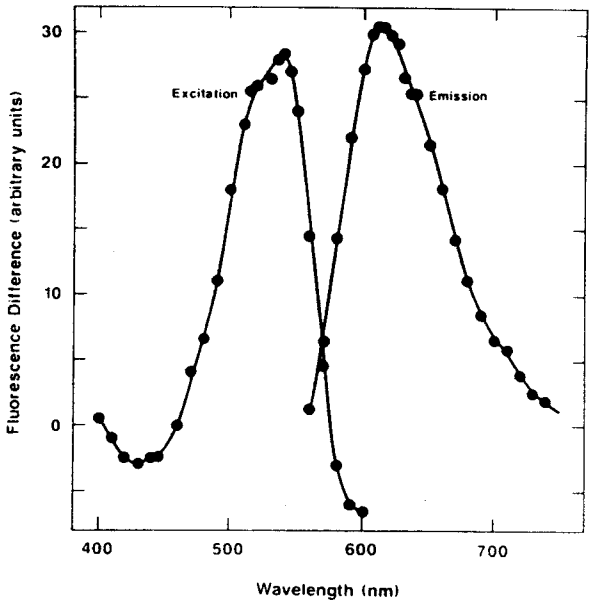

FIG. 2. Fluorescence difference spectra of propidium iodide bound to hepatocyte nuclei. Difference spectra were plotted by subtracting the sum of cell autofluorescence and propidium iodide fluorescence in the absence of cells from fluorescence in the presence of both propidium iodide- and digitonin-permeabilized hepatocytes.

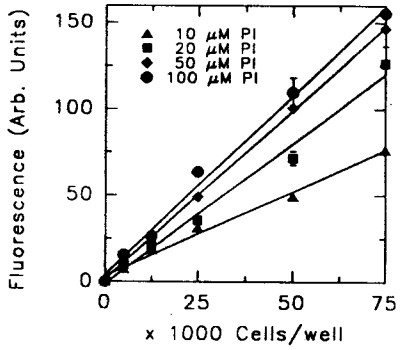

FIG. 3. Relationship of fluorescence of propidium iodide and cell number. Hepatocytes (0–75,000/well) were cultured overnight on 96-well microtiter plates as described under Materials and Methods. Culture medium was changed to Krebs–Ringer–Hepes buffer containing 10–100 μM propidium iodide (PI). Changes of fluorescence were measured after addition of 375 μM digitonin with a Pandex fluorescence scanner.

hepatocytes were exposed to 2.5 mM KCN and 0.5 mM iodoacetic acid, inhibitors of respiration and glycolysis, respectively. This treatment ("chemical hypoxia") mimics the reductive stress and ATP depletion of anoxia and produces about 50% cell killing at 45 min in freshly isolated rat hepatocytes (Gores *et al.*, 1988). We found similar kinetics of cell killing in cultured hepatocytes (Fig. 5A). About 50% of cells lost viability after 35 min.

Five-day-old cultured neonatal myocytes were exposed to 2.5 mM NaCN and 20 mM 2-deoxy-D-glucose, where 2-deoxy-D-glucose is another inhibitor of glycolysis. Cell viability measured using a fluorescence scanner decreased to 50% after 3.5 hr (Fig. 5B), similar to previous measurements in which cell killing was determined microscopically by nuclear staining (Bond *et al.*, 1991a, b).

$HgCl_2$ is a potent nephrotoxin *in vivo* (Weinberg *et al.*, 1982). In cultured rabbit proximal tubule cells, 50% loss of viability occurs after 25–35 min of exposure to 50 μM $HgCl_2$ as determined by the trypan blue exclusion assay (Smith *et al.*, 1991). By comparison, after exposure of MDCK cells, an epithelial cell line derived from a kidney, to 50 μM $HgCl_2$, 50% loss of cell viability took place after 35 min, as assessed with a fluorescence scanner (Fig. 5C).

Protection by protease and phospholipase inhibitors against cell death in cultured hepatocytes during chemical hypoxia. We evaluated protection by inhibitors of phospholipases and proteases against chemical hypoxia employing propidium iodide fluorescence. One-day cultured hepatocytes were preincubated 30 min with inhibitors at 37°C. Subsequently, 2.5 mM KCN and 0.5 mM iodoacetate were added, and cell death was assessed by propidium iodide fluorescence using a fluorescence scanner. Inhibitors of cysteine proteases (i.e., antipain, leupeptin, E-64) (Rich, 1986), serine proteases (i.e., PMSF) (Whitaker and Perez-Villasenor, 1968), and aspartic acid proteases (i.e., pepstatin A) (Umezawa and Aoyagi, 1979) did not protect against chemical hypoxia (data not shown). In contrast, 1,10-phenanthroline, an inhibitor of metalloprotease (Hiroi and Natori, 1987),

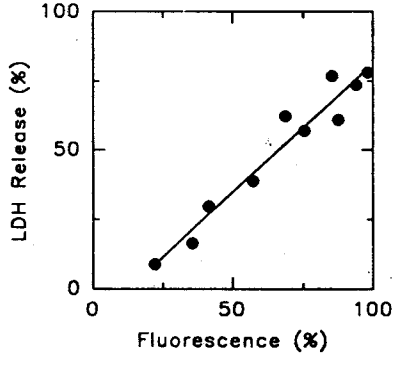

FIG. 4. Propidium iodide fluorescence and lactate dehydrogenase release by cultured hepatocytes during chemical hypoxia. Hepatocytes (50,000/well) were cultured overnight on 96-well microtiter plates as described under Materials and Methods. Culture medium was changed to Krebs–Ringer–Hepes buffer containing 50 μM propidium iodide. After 30 min preincubation, 2.5 mM KCN plus 0.5 mM iodoacetate was added. Fluorescence was measured every 15 min with a Titertek Fluoroskan II fluorescence scanner. Simultaneously, aliquots of supernatant were removed from the wells and analyzed for LDH. A 100% response of fluorescence and LDH release was taken as that after addition of 375 μM digitonin. The correlation coefficient of the least-squares regression is 0.972.

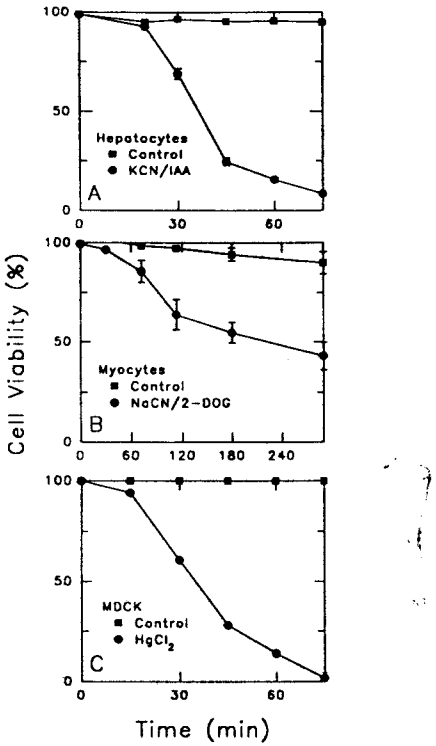

FIG. 5. Validation of cytotoxicity assay with different cell types. (A) One-day cultured hepatocytes (35,000/well) were exposed to 2.5 mM KCN and 0.5 mM iodoacetate. (B) Five-day cultured myocytes (35,000/well) were exposed to 2.5 mM NaCN and 20 mM 2-deoxy-D-glucose. (C) MDCK cells (75% confluent) were exposed to 50 μM $HgCl_2$. Cell viability over time was assessed by propidium iodide fluorescence. A CytoFluor 2300 fluorescence scanner was used in A and B, and a Titertek Fluoroskan II fluorescence scanner was used in C. Data represent a typical experiment.

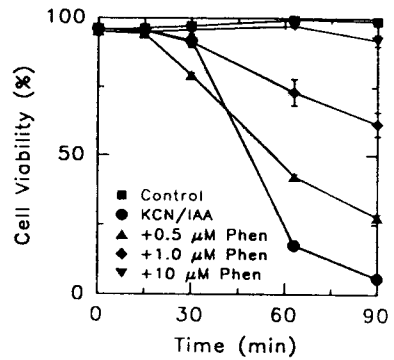

FIG. 6. Protection by 1,10-phenanthroline against chemical hypoxia in cultured hepatocytes. Hepatocytes (35,000/well) were cultured overnight on 96-well microtiter plates as described under Materials and Methods. Cells were preincubated for 30 min in the presence or absence of 1,10-phenanthroline (Phen) in Krebs–Ringer–Hepes buffer containing 30 μM propidium iodide. KCN (2.5 mM) plus 0.5 mM iodoacetate (KCN/IAA) was added at 0 min and propidium iodide fluorescence was monitored by a CytoFluor 2300 fluorescence scanner. Data represent a typical experiment.

markedly protected against the onset of cell death. Protection was dose-dependent with half-maximal protection after 60 min occurring at about 0.5 μM (Fig. 6). Iminodiacetic acid, another inhibitor for metalloprotease, had no effect on cell viability (data not shown). Phospholipase inhibitors, chlorpromazine (50 μM) and mepacrine (50 μM) (Harrison *et al.*, 1991), also substantially retarded cell killing. After 60 min of chemical hypoxia, cell viability was 94% in chlorpromazine-treated cells and 81% in mepacrine-treated cells compared to 15% without inhibitors (Fig. 7). None of these agents was toxic to hepatocytes alone at the concentrations used. Chlorpromazine by itself was toxic to hepatocytes at concentrations of 500 μM and greater (data not shown).

Effect of U74006F and calciphor on cell death in cultured hepatocytes after chemical hypoxia and t-BuOOH. U74006F is a novel steroid which protects against lipid peroxidation after ischemia (Braughler *et al.*, 1987). Thus, we evaluated U74006F for protection against cell death in the model of chemical hypoxia. One-day cultured hepatocytes were preincubated 30 min with U74006F at 37°C. Subsequently, 2.5 mM KCN plus 0.5 mM iodoacetate was added and cell death was assessed by propidium iodide fluorescence. At 50 μM U74006F, 50% cell killing occurred after 50 min compared to 75% cell killing without the inhibitor (Fig. 8A). U74006F (50 μM) also delayed cell killing caused by the oxidant, *t*-BuOOH, but the effect was small (Fig. 8B). By itself, U74006F was not toxic at concentrations up to 100 μM (data not shown).

Calciphor is a dimer of prostaglandin B_1 which provides significant improvement of mitochondrial function after renal ischemia (Widener *et al.*, 1987). However, calciphor

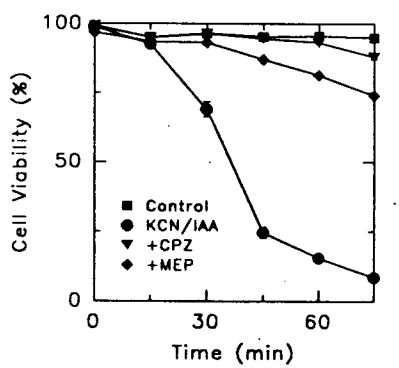

FIG. 7. Protection by chlorpromazine and mepacrine against chemical hypoxia in cultured hepatocytes. Hepatocytes (35,000/well) were cultured overnight on 96-well microtiter plates as described under Materials and Methods. Cells were preincubated for 30 min with 50 μM chlorpromazine (CPZ), 50 μM mepacrine (MEP), or no inhibitor in Krebs–Ringer–Hepes buffer containing 30 μM propidium iodide. At 0 min 2.5 mM KCN plus 0.5 mM iodoacetate was added and propidium iodide fluorescence was monitored as described in the legend to Fig. 6. Data represent a typical experiment.

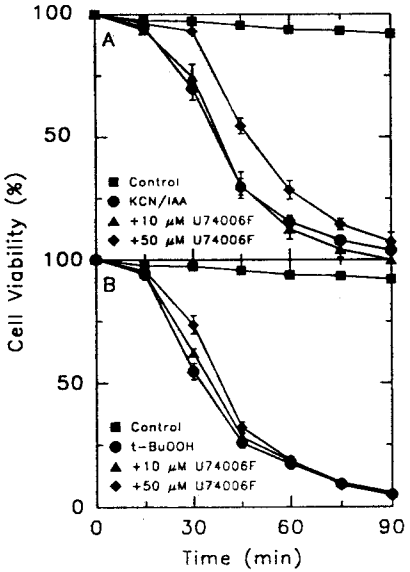

FIG. 8. Protection by U74006F against chemical hypoxia and *t*-BuOOH in cultured hepatocytes. Hepatocytes (35,000/well) were cultured overnight on 96-well microtiter plates as described under Materials and Methods. Culture medium was changed to Krebs–Ringer–Hepes buffer containing 30 μM propidium iodide. After 30 min of preincubation with U74006F, 2.5 mM KCN plus 0.5 mM iodoacetate (KCN/IAA) or 300 μM *t*-BuOOH was added at 0 min, and propidium iodide fluorescence was monitored as described in the legend to Fig. 6. Data represent a typical experiment. In these and other experiments, differences of cell viability of more than 10% were statistically significant. For example, in B after 30 min cell viability was greater in the presence of 10 or 50 μM U74006F than after treatment with *t*-BuOOH alone ($p < 0.05$ by Student's *t* test).

did not protect against cell killing during chemical hypoxia and *t*-BuOOH toxicity (Fig. 9). Calciphor by itself became toxic at concentrations ≥ 50 μM (data not shown).

DISCUSSION

The purpose of this study was to develop a simple and rapid cytotoxicity screening assay for cultured cells using a multi-well fluorescence scanner. During the course of the study, several different commercially available multi-well fluorescence scanners were evaluated. Each instrument was equipped with excitation and emission filter wheels. Filters were selected either manually or by a computer connected to the instrument. The Fluoroskan II and CytoFluor 2300 instruments were more flexible in terms of changing filters. Any combination of excitation and emission filters could be selected freely. With the Pandex instrument, excitation and emission filter pairs were fixed, and filters could not be renewed or replaced. Average scanning time for a plate was about 35 sec, which was approximately the same for the different scanners. The CytoFluor 2300 and the Pandex instruments also had an option to change photomultiplier gain. This feature was important because the fluorescence of propidium iodide was relatively weak. Results from the different instruments were, in general, equivalent, and each was employed successfully for cytotoxicity screening.

Optimal excitation and emission wavelengths for a propidium iodide-based cytotoxicity assay were determined from a difference spectrum representing the change of fluorescence caused by interaction of propidium iodide with digitonin-permeabilized cells. The maximum changes of excitation and emission fluorescence occurred at wavelengths of 540 and 610 nm, respectively (Fig. 2). With different fluorescence scanners, we were limited to filters provided by the manufacturer. Therefore, we used filter pairs which were closest to these optimal wavelengths.

With the three fluorescence scanners evaluated in this study, propidium iodide fluorescence increased with cell number between 0 and 75,000 digitonin-permeabilized cells per well (Fig. 3). Optimal propidium iodide concentration varied from 5 to 50 μM with the different scanners. The Titertek Fluoroskan II instrument was most sensitive to propidium iodide fluorescence, probably because it was equipped with a 605-nm-long pass emission filter, rather than a band-pass filter. The Pandex instrument was equipped with the least suitable emission filter (575 nm). Presumably, the per-

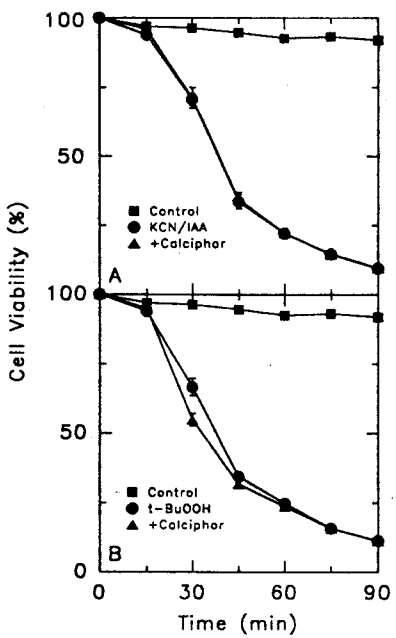

FIG. 9. Effect of calciphor during chemical hypoxia and *t*-BuOOH toxicity in cultured hepatocytes. Hepatocytes (35,000/well) were cultured overnight on 96-well microtiter plates as described under Materials and Methods. Culture medium was changed to Krebs–Ringer–Hepes buffer containing 30 μM propidium iodide. After 30 min of preincubation with 5 μM calciphor, 2.5 mM KCN plus 0.5 mM iodoacetate (KCN/IAA) or 300 μM *t*-BuOOH was added at 0 min and propidium iodide fluorescence was monitored frequently as described in the legend to Fig. 6. Data represent a typical experiment.

formance of the CytoFluor 2300 and Pandex instruments could be improved further with more suitable long-pass emission filters.

The geometry of the fluorescent light path for the Pandex and Titertek scanners differed from that for the CytoFluor instrument. In the Pandex and Titertek, light to each well came from above the plate, whereas the CytoFluor illuminated from below. With illumination from below, microtiter plates could be scanned without removing plate covers, thus preventing the risk of bacterial contamination of the cultures. A below-the-plate light path also had a theoretical advantage, because plated cells were more directly illuminated. However, in terms of the cytotoxicity assay, this did not seem to matter.

An ideal cytotoxicity screening assay should work regardless of cell type. Here, we validated the propidium iodide cytotoxicity assay in three cell types: two primary cultures and an established cell line (Fig. 5). When primary cultures of rat hepatocytes were depleted of ATP, the kinetics of cell killing were similar to that found earlier in freshly isolated hepatocyte suspensions (Gores *et al.*, 1988) and cultured cells (Harrison *et al.*, 1991). Similarly, in ATP-depleted neonatal myocytes, 50% cell killing occurred after about 3.5 hr. Earlier, similar treatment caused 50% loss of viability in 2–3 hr as assessed microscopically by propidium iodide exclusion (Bond *et al.*, 1991a, b).

We also evaluated the cytotoxicity screening assay in MDCK cells, an established epithelial cell line derived from kidney. Half-maximum cell killing occurred after 35 min of exposure to 50 μM $HgCl_2$. Smith *et al.* (1991) showed similar rate of cell killing in cultured rabbit proximal tubule cells as determined by trypan blue exclusion. Taken together, these results validate the cytotoxicity screening assay for three different cell types in monolayer cell culture under conditions of ATP depletion or chemical injury with $HgCl_2$.

The cytotoxicity assay described here monitors the breakdown of plasma membranes in cells. The inability of cells to retain cytosolic enzymes, such as LDH, reflects irreversible plasma membrane damage and is generally accepted as an indicator of cell death (Korzeniewski and Callewaert, 1983). Here, we show that propidium iodide fluorescence measured by fluorescence scanners increases proportionately with LDH release into the culture medium (Fig. 4). Although LDH release and propidium iodide fluorescence provided equivalent information, LDH assays were much more time-consuming and labor-intensive than measurements using scanning fluorometers. The observation that relative increases of propidium iodide fluorescence occurred slightly sooner than corresponding increases in LDH release indicates that propidium iodide is an earlier indicator of cell death than LDH release. Previously in suspended hepatocytes, no delay was found between increases of propidium iodide fluorescence and LDH release (Gores *et al.*, 1988). The delay of LDH release from cultured cells likely represents the time required for diffusion into the bulk medium. An additional disadvantage was that LDH assays required removal of multiple aliquots of culture medium, which the propidium iodide assay did not require. The propidium iodide assay is based on nonenzymatic binding of propidium iodide to double-stranded nucleic acid, principally the DNA of nuclei. In theory, such binding would not be effected by toxins or toxic chemicals causing enzyme inhibition or denaturation (Green *et al.*, 1984).

Release of trapped intracellular probes also reflects irreversible injury to plasma membranes. However, cells must be preloaded with such probes prior to experiments. ^{51}Cr is widely used as a cytotoxicity assay in many cell types (Spangberg, 1973). However, high intracellular concentrations of radionuclide may perturb some cell functions in long-term culture. Furthermore, ^{51}Cr is associated with proteins in membranes to different degrees. Thus, all ^{51}Cr-labeled proteins may not be released by cell lysis (Holme *et al.*, 1982). Additional disadvantages of this technique are the need for safe disposal of isotopes and the time required for counting large numbers of samples.

Retention of fluorescent probes has been used as a marker of cell viability. Such probes include fluorescein, BCECF, and Fura-2 (Jones and Senft, 1985; Lemasters *et al.*, 1987; Herman *et al.*, 1988; Kolber *et al.*, 1988). These cytosolic markers are introduced into cells as membrane-permeant esters. Inside cells, nonspecific esterases cleave ester bonds to liberate fluorescent-free acid forms which are trapped within the cell. After lysis of cell membranes and cell death, these probes leak rapidly into the extracellular medium. Loss of cellular fluorescence can then be followed as an indication of loss of viability. However, some cell types do not retain fluorescent probes well. Leakage may occur by relatively specific pathways such as the organic anion transport system (Steinberg *et al.*, 1987) and by other unidentified mechanisms. This type of leakage leads to loss of cellular fluorescence, but is unrelated to cytotoxicity.

Some cytotoxicity assays monitor disruption of intracellular organelles, such as mitochondria and lysosomes. The fluorescent cationic laser dye, rhodamine 123, accumulates electrophoretically into polarized mitochondria in response to the mitochondrial membrane potential (Emaus *et al.*, 1986). Loss of mitochondrial rhodamine 123 fluorescence after exposure to toxic chemicals indicates mitochondrial depolarization. Such loss has been used to monitor loss of viability of various cell types (Fahey *et al.*, 1989; Lachowicz *et al.*, 1989; Rahn *et al.*, 1991). Similarly, neutral red is a weak base which accumulates into the acidic lysosomal compartment, and a neutral red retention assay has been devised to monitor lysosomal integrity as a measure of viability of cells cultured in 96-well microtiter plates (Nemes *et al.*, 1979; Borenfreund and Puerner, 1984). To monitor neutral red retention, absorbed dye is extracted and measured spectrophotometrically. Rhodamine 123 and neutral red reflect integrity of mitochondria and lysosomes rather than collapse of the plasma membrane permeability barrier. Depending upon the type of injury, mitochondrial depolariza-

tion and lysosomal alkalinization may precede, follow, or be unrelated to the onset of cell death (Lemasters *et al.*, 1987; Nieminen *et al.*, 1990a, b; Bronk and Gores, 1991). Thus, release of rhodamine 123 and neutral red does not necessarily imply irreversible injury.

A colorimetric assay using MTT measures proliferation l viability of cultured cells (Mosmann, 1983). Mitochon- il dehydrogenases reduce MTT to a purple formazan ch can be quantitated spectrophotometrically. After cells viability, their mitochondria lose the ability to reduce T. Thus, like rhodamine 123 uptake, MTT reduction is ndirect indicator of cell viability whose loss may not espond exactly with the onset of cell death. MTT re- ion is also an endpoint assay which destroys the cells g measured and which requires a long incubation time ye reduction.

ir cytotoxicity screening assay provided a fast and simple od to screen cytoprotective agents. A number of hy- ses have been proposed to explain cell death after ATP tion, including protease activation, phospholipid deg- on, and free radical formation (Chien *et al.*, 1978; Far- *al.*, 1981; Adkison *et al.*, 1986). Accordingly, we ed several protease and phospholipase inhibitors and putative protective agents. We observed a striking pro- with the protease inhibitor, 1,10-phenanthroline, lethal injury in ATP-depleted hepatocytes (Fig. 6). nospholipase inhibitors, chlorpromazine and mepa- lso substantially delayed the onset of cell killing (Fig. results support the hypothesis that proteolysis and olipid degradation are critical events leading to lethal injury. Inhibitor specificities may assist in the iden- n of the many cellular proteases and phospholipases important in cell killing.

y-flow ischemia and respiratory failure, tissue injury om a relative but not absolute lack of oxygen. During tive hypoxia, oxidation-reduction components be- luced and can serve as electron donors for oxygen ormation. Previously, we observed that the antiox- annitol, desferrioxamine, and cyanidanol, delayed g in suspended hepatocytes during chemical hypoxia *al.*, 1989). This implicated involvement of oxygen- als in the mechanism of lethal injury during relative U74006F is a novel steroid that has been shown to ainst lipid peroxidation after ischemia (Braughler 7). In the present study, U74006F provided pro- ainst chemical hypoxia (Fig. 8A). U74006F also e onset of cell death after oxidant stress with *t*- ut to a lesser extent. Another potential protective , calciphor (Widener *et al.*, 1987), did not delay during chemical hypoxia or after *t*-BuOOH

sion, a simple, inexpensive, high-capacity cyto- ening assay was developed for cells cultured in rotiter plates. The assay is suitable for several ll types. Unlike other available toxicity screening assays, cells do not have to be removed from incubation medium for measurement. This makes possible continuous measurement of cell viability in a nondestructive manner. This assay appears ideal for large-scale toxicological screening of new chemical compounds emerging from the chemical and pharmaceutical industries.

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