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A Comparative Study of the Toxicity of Chemically Reactive Xenobiotics Towards Adherent Cell Cultures: Selective Attenuation of Menadione Toxicity by Buthionine Sulphoximine Pretreatment

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Abstract—Metabolic activation to reactive intermediates is a prerequisite for many forms of chemically-induced toxicity. Hepa 1c1c-9 cells were exposed to varying concentrations of several reactive metabolites implicated in adverse drug reactions and the toxicity of the compounds assessed using applied fluorescence technology. Cytotoxicity was assessed using the fluorescence of 2′, 7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein as an index of cell viability. The role of glutathione in cellular defence against these chemicals was investigated by pretreating the target cells overnight with buthionine sulphoximine, a specific inhibitor of glutathione synthesis. Depletion of intracellular glutathione augmented the toxicity of *N*-acetyl-*p*-benzoquinone imine (1·5–3-fold at 100 and 10 μM). Toxicity produced by the hydroxylamine of sulphamethoxazole (500 μM) was dependent entirely on pretreatment of the cells with buthionine sulphoximine (% cell death = 33 ± 16 compared with 0 ± 4 in untreated cells, $P < 0.05$). By contrast, the lethal effects of the model quinone, menadione, were attenuated markedly following glutathione depletion. The data obtained suggest that this assay, previously used with suspension cultures, may be useful in the rapid in-vitro screening of putative reactive intermediates. Moreover, the application of such methodology should prove beneficial for the elucidation of cellular mechanisms of defence and detoxification.

Much interest is currently focused on the development of in-vitro assays to detect xenobiotic-induced cytotoxicity and hence to predict and prevent toxicity in-vivo. The majority of drugs and environmental chemicals are themselves chemically inert from the viewpoint of toxicity and require metabolic activation to various intermediates before they can elicit their adverse effects (Monks & Lau 1988). Rodent and human cell lines are an attractive and extensively used source of target cells for such in-vitro studies and provide a useful alternative to freshly isolated hepatocytes, bypassing the need for routine preparation of fresh biological material (Wiebel et al 1984). However, the balance between activating and detoxifying biotransformations within these cells may not reflect accurately the pattern observed in-vivo (Wiebel et al 1984) and marked inter-species variation in drug metabolism (Williams 1974) often precludes a useful extrapolation of data obtained to the situation in man.

Nevertheless, the use of cell lines in mechanistic toxicology has provided a great deal of information regarding the mechanisms by which reactive metabolites of drugs and chemicals interact with vital cellular macromolecules and lead to cell damage and, ultimately, cell death (Ekwall 1983; Grisham & Smith 1984). The problem of how to portray adequately metabolic activation in man may be circumvented by incubating the target cells with varying concentrations of the pro-reactive metabolite or the ultimate toxin of the compound in question, where the identity, chemical stability and reactivity of these intermediates is known. Indeed, this approach has been implemented widely and, despite some shortcomings (Bartolone et al 1988; Riley et al 1990), has provided invaluable insights into the processes by which reactive intermediates may overcome detoxification and defence mechanisms (Monks & Lau 1988; Rieder et al 1988; Nicotera et al 1989).

In the present study, we have adopted the latter approach and attempted to apply existing fluorescence probe technology, frequently used with cell suspensions (Leeder et al 1989; Dyson 1990), to examine the effects of various chemicals on the viability of adherent cell cultures. The use of this approach with cell suspensions provides a rapid in-vitro toxicity assay with acceptable variability, small sample size requirements, and high throughput for the processing of a large number of samples or for assessing the potential toxicity of a batch of different chemicals (Leeder et al 1989). In addition, this technique avoids the need for labour intensive operator-biased light microscopy and the use of radioisotopes, which are commonly employed in viability assessment assays.

Materials and Methods

Chemicals

The acetoxymethyl ester of 2′,7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF-AM) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Anhydrous (dry; water < 0·005%) dimethylsulphoxide (DMSO) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and sulphamethoxazole, paracetamol, 2-methyl-1,4-naphthoquinone (menadione) and DL-buthionine-[*S,R*]-sulphoximine were acquired from Sigma Chemical Co. (St Louis,

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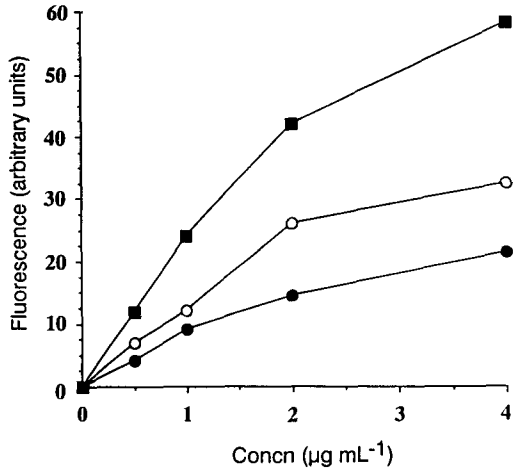

FIG. 1. Typical time and concentration dependence profiles of the loading of Hepa 1c1c-9 cells with BCECF. Ordinate values are arbitrary fluorescent units obtained with the different loading concentrations of the BCECF ester (abscissa values) after the times indicated. Points represent means of four independent determinations (s.d. < 10%). ● 15, ○ 30, ■ 60 min.

MO, USA). *N*-Acetyl-*p*-benzoquinone imine (NAPQI) and the hydroxylamine and nitroso metabolites of sulphamethoxazole (SMX-HA and SMX-NO, respectively) were synthesized by Dalton Chemical Laboratories (Toronto, Ontario, Canada). The purity of these compounds (determined by NMR) was >98%. All other chemicals were of the highest grade commercially available.

Cell culture maintenance

Hepa 1c1c-9 cells were routinely maintained as adherent monolayer cultures in α-minimum essential medium (α-MEM, University of Toronto Media Supplies, Toronto, Ontario, Canada) supplemented with 10% foetal calf serum in a 95% air–5% CO_2 atmosphere at 37°C in 75 mL flasks. Before use in toxicity studies, exponentially growing cells (60–70% confluent) were trypsinized, counted and incubated overnight in 96-well plates (Costar Packaging, Cambridge, MA, USA) at $2{\cdot}5 \times 10^4$ cells/well. Depletion of intracellular glutathione was achieved by incubating the cells overnight (for 16 h) with BSO (0·03–1·0 mM), a specific inhibitor of glutathione biosynthesis (Griffith & Meister 1979).

Fluorescence-based viability assessment

To determine optimal loading conditions for the fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), the medium was removed by aspiration and cells (5×10^4) were incubated with varying concentrations of the acetoxymethyl ester permeant form of the dye (0·5–4 μg mL^{-1}) in 0·1 M phosphate-buffered saline (PBS) for different lengths of time (15–60 min) at 37°C. Following incubation with the dye, the cells were washed once in PBS, the buffer removed to waste and the fluorescence of the cells read by front surface fluorimetry in a Screen Machine (Baxter Healthcare Corp., Pandex Division, Mundelein, IL, USA) with an excitation/emission setting of 485 nm/535 nm as previously described (Leeder et al 1989). Data collection and analysis was on an Apple Macintosh computer. Total processing time for a 96-well plate was approximately 2·5 min.

Determination of chemically-induced cytotoxicity

The relative toxicity of the compounds investigated was determined by exposing the cells ($\sim 5 \times 10^4$) to varying concentrations of these chemicals for 30 min at 37°C. The chemicals were added in DMSO (which was also added to control incubations and at the final concentration used of 1%, v/v, was not associated with any measurable toxicity) and the total incubation volume was 200 μL. This initial challenge was conducted in α-MEM (in the absence of calf serum) or PBS. Following the drug challenge, the medium was aspirated to waste and the toxicity of the compounds assessed either immediately or after a further overnight incubation in α-MEM containing 10% foetal calf serum, using BCECF fluorescence as an index of cellular viability (Leeder et al 1989) once optimal loading conditions had been established. The fluorescence of cells treated with vehicle alone was taken as 100% viability. A comparison between toxicity directed against BSO-pretreated cells and untreated controls permitted an assessment of the role of glutathione in cytoprotection against these various agents.

Results and Discussion

The aim of this investigation was to evaluate fluorescent probe technology in the study of xenobiotic-induced toxicity

Table 1. Toxicity of menadione and reactive drug metabolites towards Hepa 1c1c-9 cells assessed by BCECF fluorescence. Values are percentages of the fluorescence observed with cells incubated with vehicle alone (i.e. % control viability). For further details see Materials and Methods.

		Initial 30 min challenge		Further 16 h incubation	
Compound	Concn (μM)	Untreated	BSO-treated	Untreated	BSO-treated
Menadione	10	99±3	99±3	16±3	58±8**
	100	21±0	52±8**	0	0
NAPQI	10	98±4	98±3	83±10	35±7**
	100	51±8	33±6*	4±5	0
SMX-HA	100	99±6	99±5	99±11	99±6
SMX-NO	10	99±6	99±5	99±5	99±9
	100	99±4	99±3	99±4	99±3

Data and mean±s.d. of three experiments performed in triplicate.
* $P < 0{\cdot}05$, ** $P < 0{\cdot}001$ compared with values obtained with cells not treated with BSO.

in adherent cell cultures. Attention was focused on two chemically reactive compounds which have been studied extensively in a variety of systems, *N*-acetyl-*p*-benzoquinone imine (NAPQI), the ultimate toxin of paracetamol, and the electrophilic model quinone, menadione. The studies were conducted with a mouse hepatoma cell line (Hepa 1c1c-9) as, classically, hepatocytes have been a popular choice for mechanistic toxicity studies, primarily because the liver is the predominant site of drug biotransformation and is frequently a target for adverse drug reactions (Timbrell 1983).

Fig. 1 shows that Hepa 1c1c-9 cells readily cleaved BCECF-AM and retained BCECF.Typical working conditions for dye-loading were taken to be 2 μg mL^{-1} dye for 30 min, allowing for counts obtained, incubation time required and dye usage. Pretreatment of the target cells with BSO (1 mM for 16 h) was not associated with any measurable toxicity (data not shown), although this protocol depletes intracellular glutathione to less than 10% of control levels (Gipp et al 1991). Menadione was the most toxic compound studied (Table 1), toxicity being clearly discernible at 10 μM following a 30 min challenge and further 16 h incubation in drug-free medium (% cell death with and without prior BSO treatment = 42 ± 8 and 84 ± 3, respectively; mean $\pm$ s.d., n = 3). Neither paracetamol nor sulphamethoxazole exhibited detectable toxicity at concentrations up to 2 mM (data not shown). The pro-reactive and reactive metabolites of sulphamethoxazole, SMX-HA and SMX-NO, were toxic only at the highest concentration investigated (500 μM). At this concentration, the toxicity of SMX-HA was entirely dependent on the depletion of glutathione by BSO (% cell death = 33 ± 16 compared with 0 ± 4 in untreated cells, $P < 0.05$), whereas the degree of cell death produced by SMX-NO was too extensive (> 90%) to assess accurately the role of glutathione. When the initial challenge was performed in α-MEM, NAPQI produced a significant decrease in cell viability only at the highest concentration investigated (500 μM; data not shown), suggesting that components of the buffer (presumably low mol. wt thiols) conferred protection on the cells from this otherwise highly toxic compound. Standardized drug challenges were therefore performed in simplified medium containing no protein or low mol. wt thiols (PBS). For all the compounds, the toxicity profiles observed following an overnight incubation in drug-free medium were essentially exaggerations of those seen directly following the initial 30 min drug exposure and clearly demonstrated a lag period for the development of NAPQI and menadione toxicity at the lower concentration (10 μM).

Cytotoxicity produced by NAPQI has been an important model in defining the mechanisms by which drugs and other chemicals produce lethal injury. Although the precise pathogenesis of paracetamol hepatotoxicity is the subject of continuous debate and investigation, it is generally accepted that the complex cascade of biochemical events involves the arylation and oxidation of critical sulphydryl-containing proteins within the cell which ensues once cellular defense mechanisms involving intracellular glutathione have been overwhelmed (Monks & Lau 1988; Nelson & Pearson 1990). Furthermore, available evidence suggests that arylation may be the more lethal of these two potentially toxic insults (Nicotera et al 1989). The results of the present study support such a mechanism (see discussion below) and the toxicity of NAPQI observed was in close agreement with previous data obtained with trypan blue exclusion and isolated hepatocytes (Rundgren et al 1988; Nicotera et al 1989): NAPQI was found to be highly toxic to Hepa 1c1c-9 cells (Table 1) using BCECF fluorescence as an index of cellular viability and cell number (Leeder et al 1989) and the pivotal role of glutathione in cellular defence against NAPQI toxicity was demonstrated by the significant increase in cell death observed in cultures which had been pretreated with BSO.

Menadione is a quinone which has also been used extensively in mechanistic toxicology. As for NAPQI, the crucial biochemical events leading to menadione toxicity remain to be elucidated, although depletion of glutathione, NADPH, ATP, loss of NAD^+ and protein-sulphydryl groups, elevation of cytosolic free calcium, stimulation of the hexose monophosphate shunt, and DNA damage have all been implicated (Nelson & Pearson 1990; Redegeld et al 1990). More recently, evidence from independent sources has arisen which suggests that a depletion of intracellular adenine pools (particularly ATP) may represent a unifying hypothesis for the mechanism by which both NAPQI (Tirmenstein & Nelson 1990) and menadione (Redegeld et al 1990) mediate cytotoxicity. Menadione is highly electrophilic and may alkylate cellular macromolecules. Alternatively, enzymatic one-electron reduction to the semiquinone radical may occur within the target cells which may instil a futile redox cycle with molecular oxygen and lead to the formation of reactive oxygen species (Nelson & Pearson 1990). Although both of these mechanisms may deplete glutathione and produce toxicity, acute toxicity studies in isolated hepatocytes have suggested that alkylation of cellular macromolecules may also be the more lethal insult produced by this model quinone (Gant et al 1988). The results of the present study obtained with menadione support earlier findings that this compound is highly toxic to cells of hepatic origin (Table 1). However, pretreatment of Hepa 1c1c-9 cells with varying concentrations of BSO appeared to inhibit selectively the cytotoxicity produced by this electrophile (Fig. 2). The BSO-

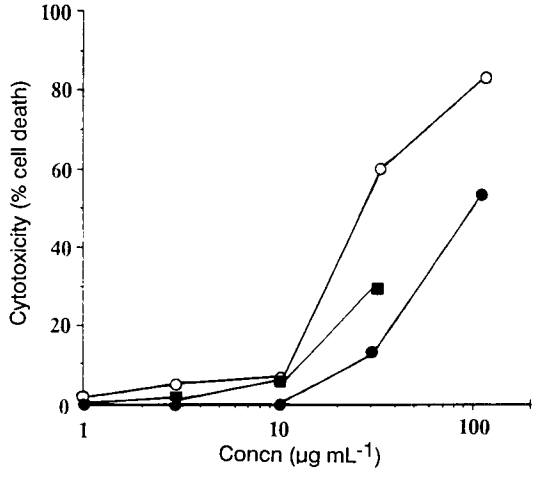

FIG. 2. Attenuation of menadione toxicity by pretreatment of Hepa 1c1c-9 cells with BSO. Points are means of triplicate determinations (s.d. < 15%) from one experiment representative of three. ○ Represents values obtained in untreated cells while closed symbols show values for cells pretreated with 30 μM (■) and 1 mM (●) BSO. Values were obtained directly after a 30 min challenge with menadione at the concentrations indicated.

dependent attenuation of menadione toxicity was observed at concentrations of BSO as low as 30 μM (Fig. 2). This finding is in direct contrast to results obtained in isolated hepatocytes where pretreatment with diethylmaleate augmented menadione toxicity (Nicotera et al 1984). However, diethylmaleate acts non-specifically as a sulphydryl reagent and affects the activity of glutathione reductase, peroxidase and other enzymes directly.

There are several possible explanations for the effect of BSO observed in the present study. Under normal circumstances, metabolic processes within the cell generate hydrogen peroxide, superoxide anions and other reactive oxygen species which are adequately detoxified by a combination of cellular defense mechanisms (Cadenas 1989). Depletion of intracellular glutathione by BSO may predispose the cell to an oxidative stress since this procedure will also deprive the cell indirectly of cytoprotective glutathione-dependent enzymes, such as glutathione transferases, peroxidase and reductase (Deneke et al 1987; Deneke & Fanburg 1989). However, since BSO pretreatment itself was not toxic, it may be inferred that the cell compensates for this oxidative stress by increasing the synthesis of a number of stress proteins (including the ubiquitous heat shock proteins) as described for bacteria (Storz et al 1990), which confer resistance to the toxic effects of menadione but not to NAPQI or SMX-HA. Indeed, the induction of such stress proteins has been observed for a number of glutathione-depleting agents with differing mechanisms of action in a variety of cell types (Loven 1988). Although a plethora of proteins may be induced, the selective protection against menadione toxicity suggests that the adverse effects of this compound may not be mediated solely through sulphydryl oxidation and arylation.

Glutathione-depleting agents may induce the activity of superoxide dismutase, catalase and DT-diaphorase (Loven 1988; Spencer et al 1991). A combination of these enzymes may selectively combat the toxic effects of menadione by detoxifying reactive oxygen species produced by redox cycling or catalysing the obligatory two-electron reduction of menadione to the hydroquinone which may be conjugated and thereby detoxified. By contrast, NAPQI is a poor substrate for DT-diaphorase (Rundgren et al 1988) and neither SMX-HA (Leeder et al 1990) nor NAPQI (Rundgren et al 1988; Nicotera et al 1989) are thought to mediate their toxic effects through redox cycling.

An alternative explanation for the protective effect of BSO-mediated glutathione depletion on menadione toxicity centres on the observation that glutathionyl conjugates of this compound auto-oxidize substantially faster than the unsubstituted naphthoquinone (Buffington et al 1989). Thus, a decrease in cellular glutathione levels may attenuate menadione toxicity by preventing conjugation of the quinone to this tripeptide which may be essential for toxic levels of redox cycling within the cell (Buffington et al 1989). The mechanistic implications of these preliminary findings warrant further investigation, particularly given that menadione has been introduced as an anticancer drug (Chlebowski et al 1985) and BSO may soon be used in long-term chemotherapy as a modulator of multi-drug resistance (Meister 1988).

In conclusion, the results of the present study show that BCECF fluorescence can be used as a reliable index of cellular viability in the rapid screening of the toxicity of reactive intermediates in adherent cells. Such studies may prove useful in the elucidation of the mechanisms involved in the regulation of the cellular repertoire of defence and detoxification processes.

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