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Screening of Hepatoprotective Plant Components using a HepG2 Cell Cytotoxicity Assay

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Abstract

Identification of the active components of plants with hepatoprotective properties requires screening of large numbers of samples during fractionation and purification. A screening assay has been developed based on protection of human liver-derived HepG2 cells against toxic damage.

Various hepatotoxins were incubated with HepG2 cells in 96-well microtitre plates (30 000 cells $well^{-1}$) for 1 h and viability was determined by metabolism of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium (MTS). Bromobenzene (10 mM) and 2,6-dimethyl-*N*-acetyl-*p*-quinoneimine (2,6-diMeNAPQI, 200 mM) had greater toxic effects than *tert*-butyl hydroperoxide (1·8 mM) or galactosamine (10 mM), reducing mean viability to 44·6 ± 1·2% (s.e.m.) and 56·1 ± 2·1% of control, respectively. Protection against toxic damage by these agents was tested using a crude extract of a known hepatoprotective Sri Lankan plant, *Osbeckia aspera*, and two pure established hepatoprotective plant compounds, (+)-catechin and silymarin (1 mg mL^{-1}). Viability was significantly improved by *Osbeckia* (by 37·7 ± 2·4%, $P < 0.05$, and 36·5 ± 2·1%, $P < 0.05$, for bromobenzene and 2,6-diMeNAPQI toxicity, respectively). Comparable values for (+)-catechin were 68·6 ± 2·9% and 63·5 ± 1·1%, and for silymarin 24·9 ± 1·4% and 25·0 ± 1·6%.

This rapid and reproducible assay should prove useful for the isolation and identification of active hepatoprotective compounds in crude plant extracts.

For many years plant extracts have been used as traditional remedies to treat a wide range of liver diseases (Wagner 1989; Thabrew & Hughes 1996). In Sri Lanka, crude aqueous extracts of plants of the *Osbeckia* family are traditional remedies for the treatment of liver disorders, particularly hepatitis. Experimental studies have shown that extracts of these plants, *Osbeckia aspera* or *O. octandra* can protect rats and mice against liver injury from carbon tetrachloride and paracetamol on both biochemical and morphological criteria (Thabrew et al 1987, 1995a; Jayathilaka et al 1989). These findings were confirmed in-vitro by studies using freshly isolated rat hepatocytes (Thabrew et al 1995b). The hepatocytes were significantly damaged by incubation for 1 h with either 0·75 mM *tert*-butyl hydroperoxide, as shown by a significant increase in release of lactic dehydrogenase (LDH) and aspartate aminotransferase (AST) (by 253% and 270%, respectively, compared with controls) into the incubation medium, along with a marked increase in lipid peroxidation (Thabrew et al 1995b). Similarly, incubation of rat hepatocytes with 10 mM galactosamine for 1 h resulted in a 25% reduction in protein synthesis, along with a 180% increase in LDH release and 170% increase in AST release into the medium, compared with controls (Thabrew et al 1995b).

Identification of the active components of such plant extracts with known hepatoprotective properties requires screening of large numbers of samples obtained during fractionation and purification processes. Because of the expense and the time involved, the use of animal models or freshly isolated hepatocytes are not convenient for this large-scale screening of material separated from crude plant extracts. We have therefore attempted to devise a reproducible microplate screening assay, based on protection of cells of the human-liver-derived HepG2 cell line (Knowles et al 1980) against toxic damage, that can be used for the rapid identification of fractions of plant extracts containing active components. We describe here such an assay and its application to the study of the cytoprotective effects of the crude aqueous extract of *Osbeckia aspera* and of two well known plant-derived hepatoprotective compounds, (+)-catechin and silymarin (Handa et al 1986; Hikino & Kiso 1988).

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Materials and Methods

Chemicals

Bromobenzene was purchased from Aldrich (Gillingham, Dorset, UK). 2,6-Dimethyl-*N*-acetyl-*p*-quinoneimine (2,6-diMeNAPQI), (+)-catechin, D-galactosamine hydrochloride and all other Analar grade chemicals were purchased from Sigma (Poole, Dorset, UK). Dulbecco's Modified Eagles medium (DMEM), foetal calf serum and trypsin were purchased from Life Technologies, Paisley, Scotland. HepG2 cells were purchased from ECACC, Porton Down, Hampshire, UK. Silymarin was a kind gift from Dr Falk Pharma GmbH, Freiburg, Germany.

Plant material

Mature leaves of *Osbeckia aspera* were collected in the field at Meetiyagoda, in southern Sri Lanka. The botanical identity of the plant was confirmed by Dr A. H. M. Jayasuriya, curator, Botanical Gardens, Peradeniya, Sri Lanka, and by Dr M. Wadwa, the Herbarium, Royal Botanical Gardens, Kew, UK. A voucher specimen has been deposited at the Herbarium,

Botanical Gardens, Peradeniya, Sri Lanka, with copies at the herbaria of the Department of Pharmacy, Kings College, London and The Royal Botanical Gardens, Kew, UK.

Preparation of the crude plant extract

Fresh leaves (200 g) were cut into small pieces and boiled under reflux for 4 h with 1·6 L distilled water. The final volume was reduced to 200 mL by boiling without reflux—this being the procedure traditionally used by Ayurvedic practitioners in Sri Lanka to prepare an extract for administration to man. After filtration to remove any large particulate debris then centrifugation of the filtrate, the aqueous extract obtained was freeze-dried and the resulting powder stored at −20°C until use.

Selection and preparation of hepatotoxins

Preliminary experiments were performed with a number of known hepatotoxins to determine which would induce significant cell damage within a sufficiently short period of time to provide the basis for the hepatoprotection assay. Thus, HepG2 cells were initially incubated with D-galactosamine hydrochloride, *tert*-butyl hydroperoxide, bromobenzene or 2,6-diMeNAPQI (an analogue of the active metabolite of paracetamol) to determine which of these would reproducibly induce the required amount of cell damage within 1 h, as assessed by cell viability as described below. Solutions of these agents were prepared as follows.

D-Galactosamine hydrochloride was prepared immediately before use as a stock solution (800 mM) in distilled water adjusted to pH 7·4 with 5 mM NaOH; this was added to the incubation medium.

A 1 M stock solution of *tert*-butyl hydroperoxide in dimethylsulphoxide was prepared and diluted to 10 mM with DMEM before addition to the incubation medium.

A 1 M stock solution of bromobenzene in dimethylsulphoxide was prepared and diluted to 0·2 M with DMEM before addition to the incubation medium.

A 6 mM stock solution of 2,6-diMeNAPQI in dimethylsulphoxide was prepared immediately before use. This solution was diluted further to 2 mM with DMEM before addition to the incubation medium.

Cell culture and induction of cytotoxicity

HepG2 cells were routinely grown and sub-cultured in monolayers in DMEM supplemented with 10% (v/v) foetal calf serum. Preliminary investigations with cells at different phases of the cell cycle established that the effects of the toxins were greatest in mature HepG2 cells. Thus, all experiments in this investigation were conducted with cells that had been initially batch-cultured for 10 days. At this stage cells were harvested and plated (eight replicates per test) at approximately 30 000 cells $well^{-1}$ in 96-well microtitre plastic plates (Nunclon D, Life Technologies) and left to rest for 24 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were then exposed for 1 h to various concentrations of each of the above toxins or (as a control) medium alone. At the end of this period, cytotoxicity was assessed by estimation of the viability of the HepG2 cells by means of the Cell Titre 96 aqueous non-radioactive cell proliferation assay (Promega, Southampton, Hampshire, UK) which is based on the production of a coloured formazan derivative by reaction between a tetrazolium inner salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium (MTS), and the electron coupling reagent phenazine methosulphate (PMS). After 1 h incubation with the toxin, the medium in each well was removed by aspiration and replaced by the same volume (200 μL) of fresh medium before addition of 20 μL of the MTS/PMS solution. After 2 h incubation at 37°C, absorbance at 490 nm was determined with an ELISA plate reader (Dynatech MR 5000, Billingshurst, West Sussex, UK). In preliminary experiments it was found that there seemed to be some component(s) in the *Osbeckia* extract that can directly reduce the tetrazolium dye, giving rise to false positive results. It is thus important to replace the medium containing the plant material with fresh medium in the wells before addition of the MTS reagent.

Assessment of the cytoprotective effects of plant components

Cytoprotection by the crude aqueous extract of *Osbeckia aspera*, (+)-catechin and silymarin was assessed by estimating HepG2 cell viability as described above, after exposure to the various toxins in the presence or absence of a range of concentrations of the plant components. Results are expressed as percentage protection, i.e. the percentage increase in cell viability compared with the viability of cells treated with hepatotoxin alone. Six experiments were performed for each with eight replicate wells.

Statistical analysis

Results are expressed as means ± s.e.m. Significant differences between groups were determined using either the Mann-Whitney *U*-test or Duncan's multiple range test, as appropriate.

Results

Effects of hepatotoxins on the viability of HepG2 cells

Of the four toxins tested at a range of different concentrations, 10 mM bromobenzene and 200 μM 2,6-diMeNAPQI had the

Table 1. Effect of incubation for 1 h with different hepatotoxins on the viability, determined using MTS, of HepG2 cells.

Hepatotoxin	Concentration (mM)	Viability (% control)
tert-Butyl hydroperoxide	1.8	71.5 ± 4.2†
D-Galactosamine	10	93.2 ± 1.8
Bromobenzene	10	44.6 ± 1.2*
2,6-Dimethyl-*N*-acetyl-*p*-quinoneimine	0.2	56.1 ± 2.1*

Mean of six experiments (eight replicate wells in each) ± s.e.m. *$P < 0.01$, †$P < 0.05$, significantly different compared with controls without addition of hepatotoxin; Mann-Whitney *U*-Test.

greatest toxic effects in 1 h, reducing cell viability to 46·2 ± 1·2% and 56·1 ± 2·1%, respectively ($P < 0{\cdot}01$), of that of the control cells cultured without the toxins (Table 1). 1·8 mM *tert*-butyl hydroperoxide had a similar cytotoxic effect (46·4 ± 3·2%) but only after increasing the incubation time to 90 min; galactosamine was only weakly cytotoxic (92·4 ± 2·6% at 10 mM) and only after 90 min. As bromobenzene and 2,6-diMeNAPQI both induced significant hepatocellular damage in 1 h, and different mechanisms are involved in their cytotoxic actions (Albano et al 1985; Duthie et al 1994), it was decided to assess both of these toxins in the experiments designed to determine the protective activities of Osbeckia extract, (+)-catechin and silymarin.

Cytoprotective effects of the plant components

Different concentrations of the *Osbeckia* extract, (+)-catechin and silymarin had significant dose-dependent protective effects on the cytotoxicity of bromobenzene to HepG2 cells (Fig. 1). A similar amount of protection was obtained for the cytotoxicity of 2,6-diMeNAPQI (Fig. 2). With either agent the amount of cytoprotection was in the order (+)-catechin > *Osbeckia* > silymarin. For example, at the highest final concentration (1 mg mL^{-1}) used in the incubation medium, *Osbeckia* significantly ($P < 0{\cdot}05$) improved cell viability by 37·7 ± 2·4% and 36·5 ± 2·1%, respectively, for bromobenzene and 2,6-diMeNAPQI toxicity. Comparable values for (+)-catechin were 68·6 ± 2·9% and 63·5 ± 1·1% ($P < 0{\cdot}05$), and for silymarin, 24·9 ± 1·4% and 25 ± 1·6% ($P < 0{\cdot}05$).

Discussion

In-vitro systems based on cultured immortalized hepatoma cell lines from man are widely used for studies on toxicity, xenobiotic metabolism and carcinogenesis (Grisham 1979; Duthie et al 1988, Donato et al 1992; Jover et al 1994). During the past decade there has been increasing use of HepG2 cells for such studies, in preference to primary cultures of hepatocytes from animals or man, because of the ready availability and ease of cryopreservation and culture of this cell-line. The use of cells from man rather than animals not only avoids the killing of animals, but has the further advantage that possible species differences in responses, both to hepatotoxins and to the plant extract, are avoided. HepG2 cells retain many of the morphological and biochemical characteristics of normal hepatocytes (Knowles et al 1980; Bouma et al 1989), although it is accepted that no transformed cell-line can be an exact substitute for in-vivo studies in man or for using isolated hepatocytes from man. To the best of our knowledge, the current studies are the first use of this cell line to investigate the hepatoprotective effects of crude plant extracts.

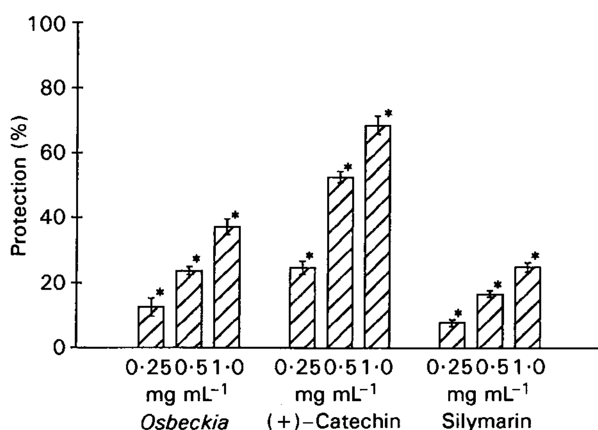

FIG. 1. Dose-dependent protective effects of plant components against damage to HepG2 cells induced by bromobenzene. Results are means from six experiments (eight replicate wells in each) ± s.e.m. *$P < 0{\cdot}05$ compared with hepatotoxin alone; Duncan's multiple-range test.

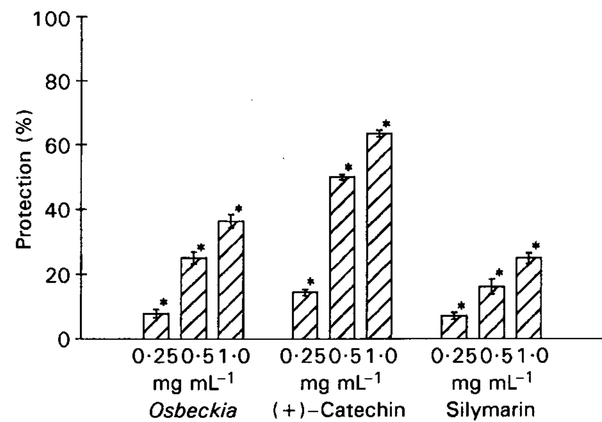

FIG. 2. Dose-dependent protective effects of plant components against damage to HepG2 cells induced by 2,6-diMeNAPQI. Results are means from six experiments (eight replicate wells in each) ± s.e.m. *$P < 0{\cdot}05$ compared with hepatotoxin alone; Duncan's multiple-range test.

In this investigation concentrations of *tert*-butyl hydroperoxide and galactosamine similar to those used in earlier experiments with isolated rat hepatocytes (Thabrew et al 1995b) caused only small reductions in viability (28·5% and 6·8%, respectively) as determined by the metabolic reduction of MTS in HepG2 cells. Although these results suggest that HepG2 cells are more resistant to these two compounds than are freshly isolated hepatocytes, they might be a reflection of differences in the assays used to assess cytotoxicity, because release of cytoplasmic enzymes might be a more sensitive marker of cell damage than metabolism of MTS, which is considered to be a measure of cellular reductive activity involving NADH- and NADPH-dependent mechanisms (Berridge & Tan 1993). However, in a previous study of the hepatotoxic effects of ethanol on HepG2 cells a similar effect on viability was observed when assessed either by metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (similar to MTS) or LDH release (Neuman at al 1993). On the other hand, the reduction in cell viability observed with 2,6-diMeNAPQI in the current study was comparable with that previously noted in our studies using a very similar dose of this toxin with isolated hepatocytes (56·1 ± 2·1% compared with 47·2 ± 5·8%), in which viability was assessed by trypan blue exclusion and cytotoxicity by measuring glutathione levels and LDH release (Thabrew et al 1995a).

The ability of the crude aqueous extract of *Osbeckia aspera* to protect HepG2 cells against bromobenzene- and 2,6-diMeNAPQI-mediated cytotoxicity provides further evidence in favour of the therapeutic potential of this plant extract in man. Results obtained in this investigation indicate that the crude *Osbeckia* extract has hepatoprotective activity intermediate

between the protective activities of the specific plant compounds silymarin and (+)-catechin. The antioxidant properties of these two compounds are thought to be responsible for their beneficial effects against liver damage (Valenzuela et al 1986; Scott et al 1993). Preliminary experiments have demonstrated free-radical scavenging effects of the *Osbeckia* extract which were intermediate between those of (+)-catechin and silymarin (Thabrew et al 1996). A similar relationship in activity was found for the protective effects on HepG2 cells against damage, suggesting that antioxidant properties might be important in the protective effects observed in the current study. It was interesting that the effects observed were similar with both hepatotoxins used; this might indicate that similar final damaging oxidative reactions are involved in both cases.

The assay described here proved to be robust, rapid and highly reproducible. Because many preparations of potentially hepatoprotective agents can be tested simultaneously in 96-well microtitre plates, it should be useful for primary screening for hepatoprotective activities of other crude plant extracts and for the large-scale screening of fractions separated from such extracts during the isolation and identification of the active components. An additional advantage over other techniques is that only small amounts of material are required. The assay can also be used to determine the effects of cytoprotective agents on other parameters including LDH release, and protein or DNA synthesis by measurement of ^{14}C-labelled amino acids or [^{3}H]thymidine incorporation, respectively.

Acknowledgements

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