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Hormesis: a stress response in cells exposed to low levels of heavy metals

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Cytotoxicity studies using a 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT)-based *in vitro* toxicity assay revealed that McCoy cells exposed to low concentrations of mercuric (0.7 μM), cadmium (1 μM) and cupric chloride (3 μM) exhibited significant increases in cellular activity.
This increased activity, previously termed hormesis, coincided with the production of high levels of the stress proteins, heat shock protein 70 (Hsp 70) and metallothionein, while the high constitutive expression of these proteins in cadmium-resistant mutant (CRM) cells corresponded to constitutive hormetic activity.
Hormesis was found to obey uniform kinetics allowing for a mathematical description of this increased activity.
These results suggest that hormetic activity is a specific cellular response, and most likely, a stress response to low but harmful levels of toxic agents and may therefore provide a rapid test for the presence of toxicants at concentrations associated with chronic toxicity. *Human & Experimental Toxicology* (2000) **19**, 420–430.

Keywords: hormesis; heavy metal; toxicity; stress response

Introduction

In 1888, Schulz observed that yeast exposed to a variety of toxic agents at low concentrations exhibited increased levels of respiration. These findings ultimately led to the establishment of the Arndt–Schulz law which stated that substances capable of inhibiting biological processes at sublethal concentrations could be expected to stimulate them at lower levels.[1] At the time, this hypothesis became generally accepted and was used as one of the scientific principles in support of homeopathic medicine.[2] In 1982, Stebbing referred to the stimulation of growth in organisms by low levels of inhibitors as hormesis. He suggested that this phenomenon could be the result of over-corrections by cellular control mechanisms to low levels of inhibition resulting in higher than normal growth or activity.[1]

More recent toxicity studies, however, have shown that daphnids exposed to low, specific concentrations of copper and cadmium initially exhibited increased activity, whereafter a significant decrease in the survival rate of test organisms was found to occur with longer exposure to these initially stimulatory levels of metal.[3]

It was therefore important to determine whether hormesis was the result of non-specific inhibition or a specific cellular response and thus a possible indicator of low, but detrimental, concentrations of toxic metals.

In this report, we present cytotoxicity studies using a sensitive 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT)-based *in vitro* toxicity assay. We show that McCoy cells exposed to low concentrations of heavy metals exhibit significant increases in cellular activity (as determined by the degree of MTT reduction) and this hormetic response coincides with the production of high levels of the stress protein, heat shock protein 70 (Hsp 70), as well as metallothionein while the high constitutive expression of these stress proteins in cadmium-resistant mutant (CRM) cells corresponds to constitutive hormetic activity.

Furthermore, we show that hormesis obeys uniform kinetics, allowing for a mathematical description of this increased activity, making it potentially useful as a specific indicator of toxicant concentrations capable of inducing a stress response in cells and therefore providing a potentially rapid test for the presence of heavy metal toxicants at concentrations associated with chronic toxicity.

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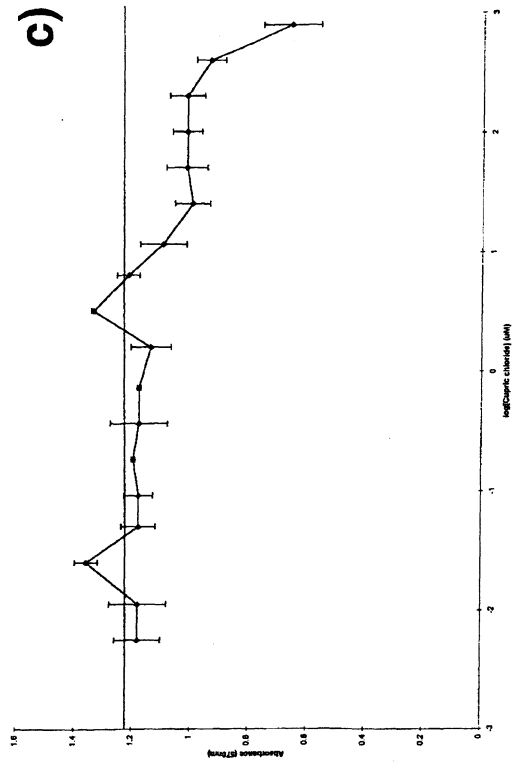
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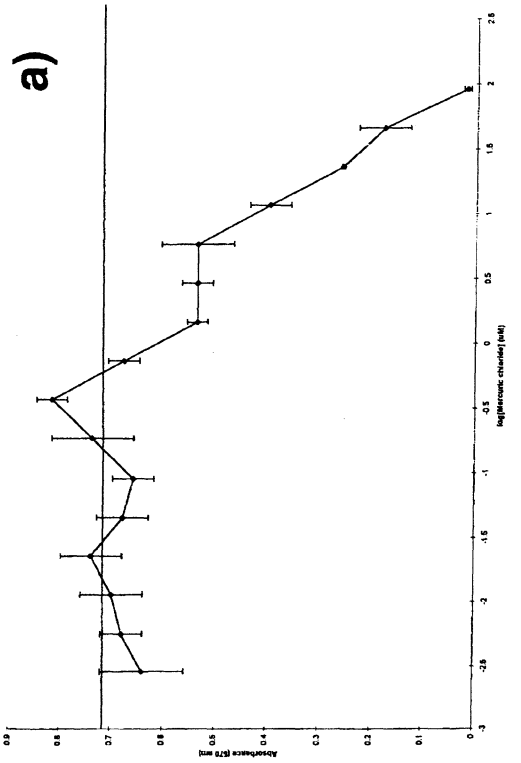
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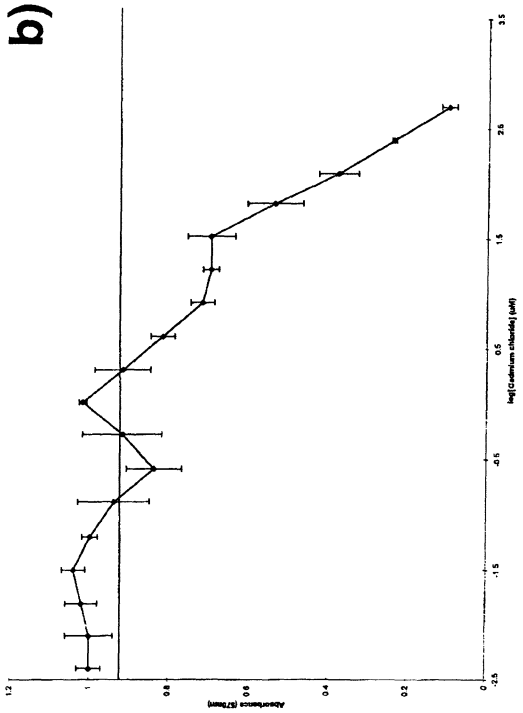
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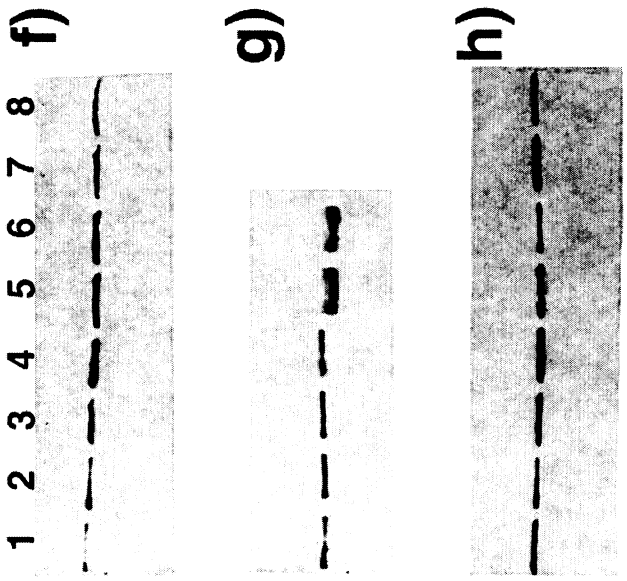
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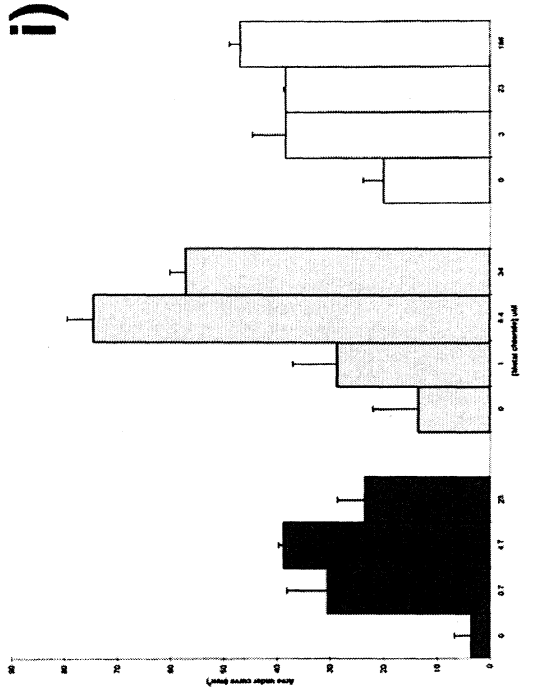
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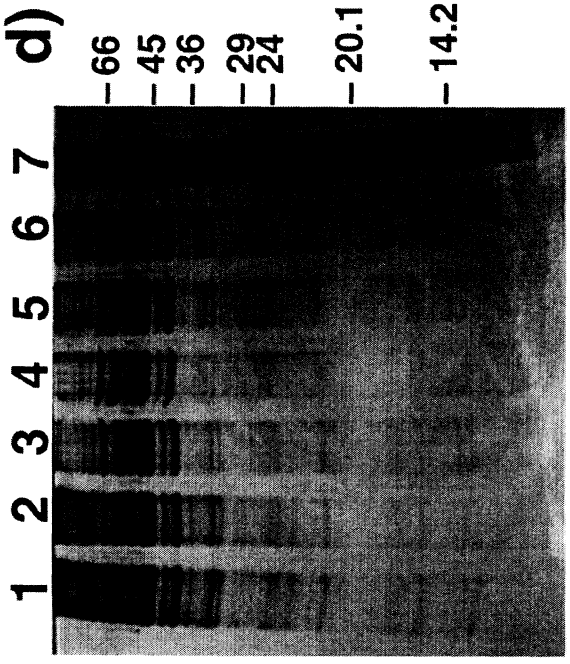
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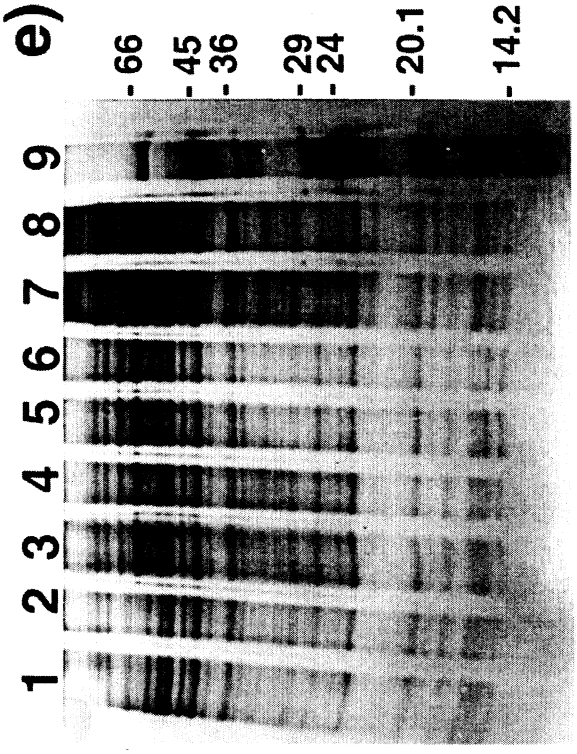
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Methods

Cell lines and culture
McCoy mouse cells, chosen for their ease of maintenance and high MTT reduction rate, were obtained from Highveld Biological Association and grown in MEM supplemented with 5% fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin (Highveld Biological) at 37°C. Cell cultures were split every 3 days in a 1:6 ratio using 0.25% w/v trypsin and 0.1% w/v ethylene diamine tetra-acetic acid (EDTA) solutions in a 1:1 ratio to detach cells from culture flasks.

Establishment of a Cadmium-resistant mutant cell line
A McCoy CRM cell line was also established. McCoy cells were grown in MEM supplemented as above until 80% confluency was obtained. Cells were then given fresh medium with 1.5 μg/ml phenazine methosulphate (Sigma) and incubated at 37°C for 18 h. Surviving cells where given fresh medium with 4.3 μM cadmium chloride and incubated for 2 weeks at 37°C. CRM cells were then maintained and cultured as above but in MEM supplemented with 8.7 μM cadmium chloride.

MTT colorimetric assay
McCoy cells and CRM cells trypsinized as above were seeded into 96-well tissue culture plates (Nunclon) at 8000 cells per well in 100 μl MEM supplemented as above. Plates were incubated for 18 h at 37°C and 5% CO_2 in air. Heavy metal salt solutions to be tested were twofold serially diluted in a master plate with MEM and 100 μl of each dilution was then further reduced to 50% of its original concentration when transferred to a corresponding well of the tissue culture plate. The cells were then incubated as before for a further 24 h. Following incubation, 10 μl of a 5 mg/ml solution of MTT (Sigma) in 0.01 M phosphate-buffered saline (PBS) was added to each well and the incubation continued for an additional 4 h. Cells were then solubilized by the addition of 10 μl 3 N HCl and 10 μl of 10% NP-40 solution (Unilab) to each well. Following this, the formazan precipitate was solubilized by the addition of 25 μl of Weaver solubilization reagent (Highveld Biological). Plates were stored at 4°C for 18 h and read on a Biorad ELISA plate reader using a 570-nm filter. The absorbance results were plotted using non-treated control samples to determine the 100% activity level.

Time/activity assays
The relationship between the onset of hormetic activity and metal concentration was determined by MTT assays described above using cupric, mercuric or cadmium chloride at concentrations of 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml. Plates were incubated for 12 h after which 10 μl of a 5 mg/ml solution of MTT in PBS was added to successive wells at 72-min intervals over a further 12-h period. Solubilization of cells in successive wells was carried out 2 h after each MTT addition.

Protein profiles
Cells were grown in 25 cm^2 flasks in MEM supplemented as above until 60% confluency was obtained. Normal cells were then fed with 10 ml fresh MEM spiked with 0.7, 4.7 and 23 μM mercuric chloride and 3, 23 and 186 μM cupric chloride while both normal and CRM cells were given 10 ml MEM with cadmium chloride at 1, 8.4 and 34 μM, respectively, corresponding to hormetic, plateau and LC_{50} concentrations for each metal. Additional flasks exposed to medium free of toxicant served as controls.

After 24 h at 37°C, cells were washed twice with 2 ml ice-cold PBS and lysed by the addition of 1 ml lysis buffer, containing 150 mM sodium chloride, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate and 0.02% sodium azide in 50 mM Tris–Cl solution (pH 8.0). After incubation on ice for 20 min, cell lysates were transferred to eppendorf tubes and centrifuged at 12,000×*g* for 3 min at 4°C in a microfuge. Supernatants were then stored at −20°C until further use. Protein concentrations were determined using the bicinchoninic acid (BCA) method as described previously.[4]

Protein profiles for cells exposed to metal chlorides were obtained by SDS polyacrylamide gel electrophoresis modified for the detection of Hsp 70 and metallothionein by Hatayama *et al.*[5] and protein bands were visualized with silver staining using a quick silver staining kit (Amersham).

Figure 1 (a, b, c) Cytotoxicity curves for mercuric chloride, cadmium chloride and cupric chloride, respectively. Untreated controls are represented by solid lines on each figure. (d and e) Protein profiles of cells exposed to hormetic, plateau and LC_{50} concentrations of mercuric and cadmium chloride for 24 h. Lanes 1, 2D and E: controls; 3, 4D and E: 0.7 μM $HgCl_2$ and 1 μM $CdCl_2$, respectively; 5, 6D: 4.7 μM $HgCl_2$; 5, 6E: 8.4 μM $CdCl_2$; 7, 8E: 34 μM $CdCl_2$; 7D and 9E: molecular weight markers (kDa). (f, g, h) Western blots showing Hsp 70 expression in cells exposed to mercuric, cadmium and cupric chloride, respectively, for 24 h. Lanes 1, 2f, g and h: controls; 3, 4f: 0.7 μM $HgCl_2$; 3, 4g: 1 μM $CdCl_2$; 3, 4h: 3 μM $CuCl_2$; 5, 6f: 4.7 μM $HgCl_2$; 5, 6g: 8.4 μM $CdCl_2$; 5, 6h: 23 μM $CuCl_2$; 7, 8f: 23 μM $HgCl_2$ and 7, 8h: 34 μM $CuCl_2$. (i) Hsp 70 levels in cells exposed to $HgCl_2$ (black bar), $CdCl_2$ (dotted bar) and $CuCl_2$ (white bar).

Quantification of Hsp 70

Hsp 70 was identified and quantified by Western blotting and enhanced chemiluminescence (ECL). Proteins separated on 12% (w/v) polyacrylamide gels as described previously[5],were transferred electrophoretically to ECL nitrocellulose membranes (Amersham) using a Hoefer Western blotting apparatus at a constant voltage of 45 V for 16 h at 4°C. After protein transfer, filters were dried on blotting paper at room temperature for 30 min, rehydrated in deionized water and stained in a 0.2% (w/v) solution of ponceau S (Merck) containing 3% (w/v) trichloroacetic and sulphosalicylic acids (Merck) in order to visualize transferred proteins. Filters were then washed in four changes of deionized water and incubated for 1.5 h at room temperature in 15 ml blocking solution containing 5% (w/v) non-fat dried milk, 0.01% (v/v) Antifoam A (Sigma) and 0.02% sodium azide in PBS.

After blocking, filters were transferred to fresh blocking solution containing a dilution anti-bovine Hsp 70 monoclonal antibody (Sigma) at a dilution of 1:3000 and incubated for 2 h at room temperature. Filters were then washed 3×10 min in 250 ml PBS and once for 10 min in 200 ml Tris–saline (150 mM NaCl, 50 mM Tris–Cl pH 7.5), then transferred to 15 ml blocking solution containing 5% non-fat dried milk and 0.01% (v/v) Antifoam A in Tris–saline with horseradish-peroxidase-coupled goat anti-mouse polyclonal antibody (Binding Site, UK) at a dilution of 1:500 and incubated at room temperature for a further hour.

Filters were then washed 4×10 min in 200 ml Tris–saline, incubated for 1 min in an equal volume of ECL detection reagents (Amersham) and exposed to Curix X-ray film (Agfa) for 1–5 min. Band intensity was quantified using laser densitometry and expressed as area under curve (mm^2).

Quantification of metallothionein

Normal and CRM cells were exposed to 1, 8.4 and 34 μM cadmium chloride for 24 h and 1 μM cadmium chloride for 0, 8, 16 and 24 h as described above.

After incubation, cells in flasks were washed twice with 5 ml ice-cold PBS, harvested with a cell scraper, resuspended in 1 ml 10 mM Tris–Cl, 85 mM Nacl (pH 7.4) and transferred to eppendorf tubes. Cells were then lysed by rapid freezing and thawing (five times) in liquid nitrogen and the resultant lysate was centrifuged at 100,000×*g* for 1 h at 4°C in a Beckman TL 100.2 ultracentrifuge.

The BCA method was used to quantify the protein concentrations of the supernatants and these were stored until further use at −70°C. Metallothionein in supernatants was then quantified and compared to levels in untreated controls by the Cd–Chelex assay as described previously.[6]

Analysis and presentation of results

All assays were performed in triplicate. Where shown, error bars on graphs indicate the standard deviations for values obtained from these triplicate assays, while for Figure 4d, the least squares line of log[cupric chloride] μg/ml vs. $b(\mu g^{-1}\ ml\ days^{-1})$ was obtained using Microsoft Excel.

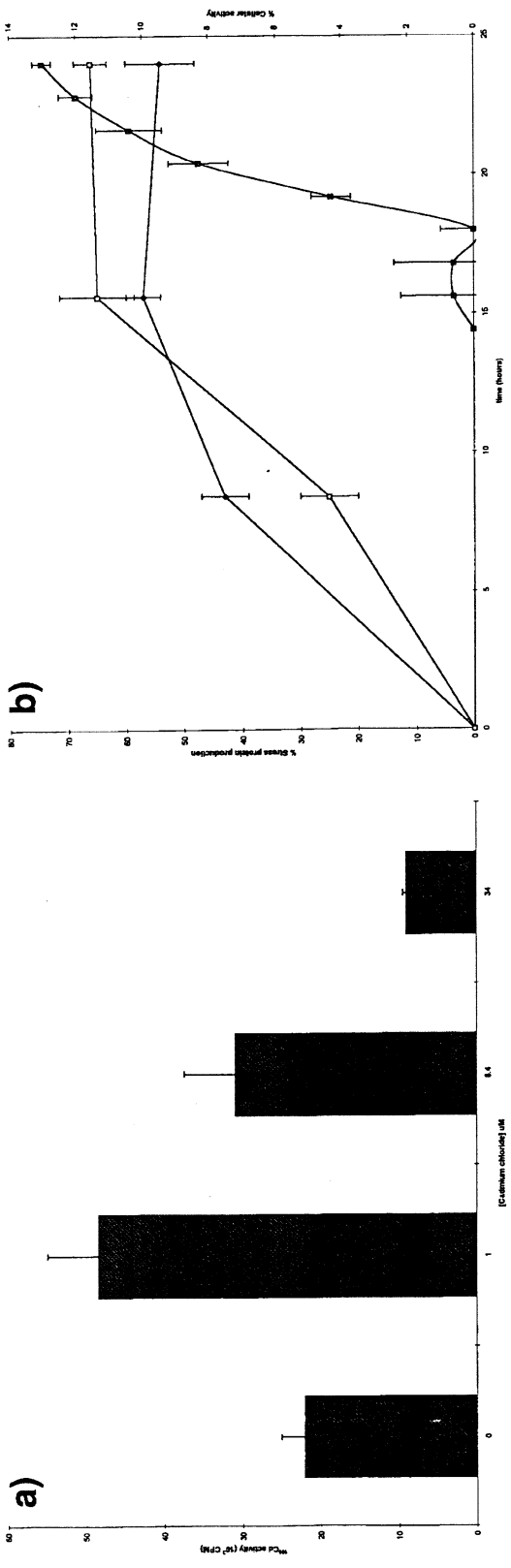
Results

The cytotoxicity curves of absorbance vs. log metal chloride for normal cells exposed to mercuric, cadmium and cupric chloride over 24 h exhibited similar characteristics and consisted of one or more peaks with activities at least 10–15% greater than that exhibited by non-treated control cells, occurring within a metal concentration range of 1 nM to 1 μM (Figure 1a, b and c). These hormetic peaks were followed by plateaux or troughs with activities at or below that of the control. The succession of peaks and troughs was then followed by a steady decline in activity with increasing metal concentration.

The protein profiles of cells exposed to mercuric, cadmium and cupric chloride for 24 h at concentrations corresponding to control, hormetic, plateau and lethal concentration 50% (LC_{50}) regions of the cytotoxicity curves for each metal salt exhibited pronounced protein bands clearly visible at and above the 66 kDa molecular weight marker when compared to the profiles of untreated control cells (Figure 1d and e). Western blotting identified the 70-kDa protein as Hsp 70, the highly conserved stress-induced protein which was found to be at levels which were twofold in cells exposed to hormetic concentrations of cadmium and cupric chloride (1 and 3 μM, respectively), and 10-fold in cells exposed to hormetic concentrations of mercuric chloride (0.7 μM) when compared to untreated controls (Figure 1f, g, h and i).

The Cd–Chelex assay is restricted to the quantification of zinc- and/or cadmium-induced metallothionein.[6] We therefore investigated metallothionein production in cells exposed to hormetic levels of cadmium chloride (1 μM) for 24 h. We found these metallothionein levels to be more than double that of untreated controls, but unlike Hsp 70, began to decline at plateau levels (8.4 μM) and then dropped below control levels at 34 μM (LC_{50}) (Figure 2a).

Over 8, 16 and 24 h, cells exposed to the hormetic concentration (1 μM) of cadmium chloride exhibited an almost hyperbolic and sigmoidal increase in Hsp 70 and metallothionein levels, respectively, with



maximum production of both proteins reached after 16 h, and maintained at those levels up until 24 h (Figure 2b). Time/activity assays for cells exposed to 1 μM cadmium chloride showed that the onset of increased activity occurred after 20 h and reached a maximum at 24 h (Figure 2b). Thus, hormesis did not only correspond to significantly increased levels of stress protein, but more specifically, the onset of peak activity corresponded to maximum Hsp 70 and metallothionein production.

Since increased levels of Hsp 70 production at hormesis strongly indicated that this phenomenon was a stress response in cells exposed to sublethal levels of toxic metals, we studied the effect of metal resistance on cellular activity and protein production in McCoy CRM cells.

We found that the cytotoxicity curve for CRM cells displayed an oscillating level of activity over the entire concentration range of cadmium chloride with a decline in activity only apparent at very high metal concentrations (34 μM) (Figure 3a). CRM cells exposed to MEM in the absence of cadmium chloride were also found to exhibit high constitutive levels of both Hsp 70 and metallothionein when compared to the controls of normal cells. Hsp 70 levels in CRM cells dropped slightly, but remained significantly high after exposure to hormetic (1 μM) and plateau (8.4 μM) concentrations of cadmium chloride and then rose to higher levels again at the LC_{50} concentration (34 μM) (Figure 3b), while metallothionein levels in these cadmium-resistant cells remained high and almost constant for all three concentrations (Figure 3c).

In addition, both Hsp 70 and metallothionein in CRM cells exposed to 1 μM cadmium chloride for 8, 16 and 24 h exhibited plateau protein levels, which corresponded to the high constitutive activity of these cells over the same time period (Figure 3d), thus clearly showing that stress protein production in CRM cells was both high and constitutive and corresponded to the constitutive hormetic activity displayed by these cells.

In order to determine the dependence of the onset of hormetic activity on metal concentration and

Figure 2 (a) Metallothionein levels in control cells, cells exposed to hormetic (1 μM), plateau (8.4 μM) and LC_{50} (34 μM) concentrations of $CdCl_2$. Protein samples (36 μg/ml) were incubated with 20 μCi/ml $^{109}CdCl_2$ (1000 μCi/μg Cd) (Amersham) for 10 min at 25°C, after which excess ^{109}Cd was removed with Chelex 100 (Biorad). Aliquots (200 μl) were then chromatographed on Sephadex G-50 column (1.0×20 cm^2) and fractions analysed for ^{109}Cd activity and compared to a purified metallothionein standard (Sigma) (not shown). (b) Time/activity assay and corresponding stress protein levels for cells exposed to 1 μM $CdCl_2$ over 24 h. Cellular activity and protein levels are relative to that of untreated controls. (diamond) Hsp 70 (peak area mm^2), (square) metallothionein (^{109}Cd activity 10^3 cpm), (shaded square) percent cellular activity expressed as percent absorbance (570 nm) above control.

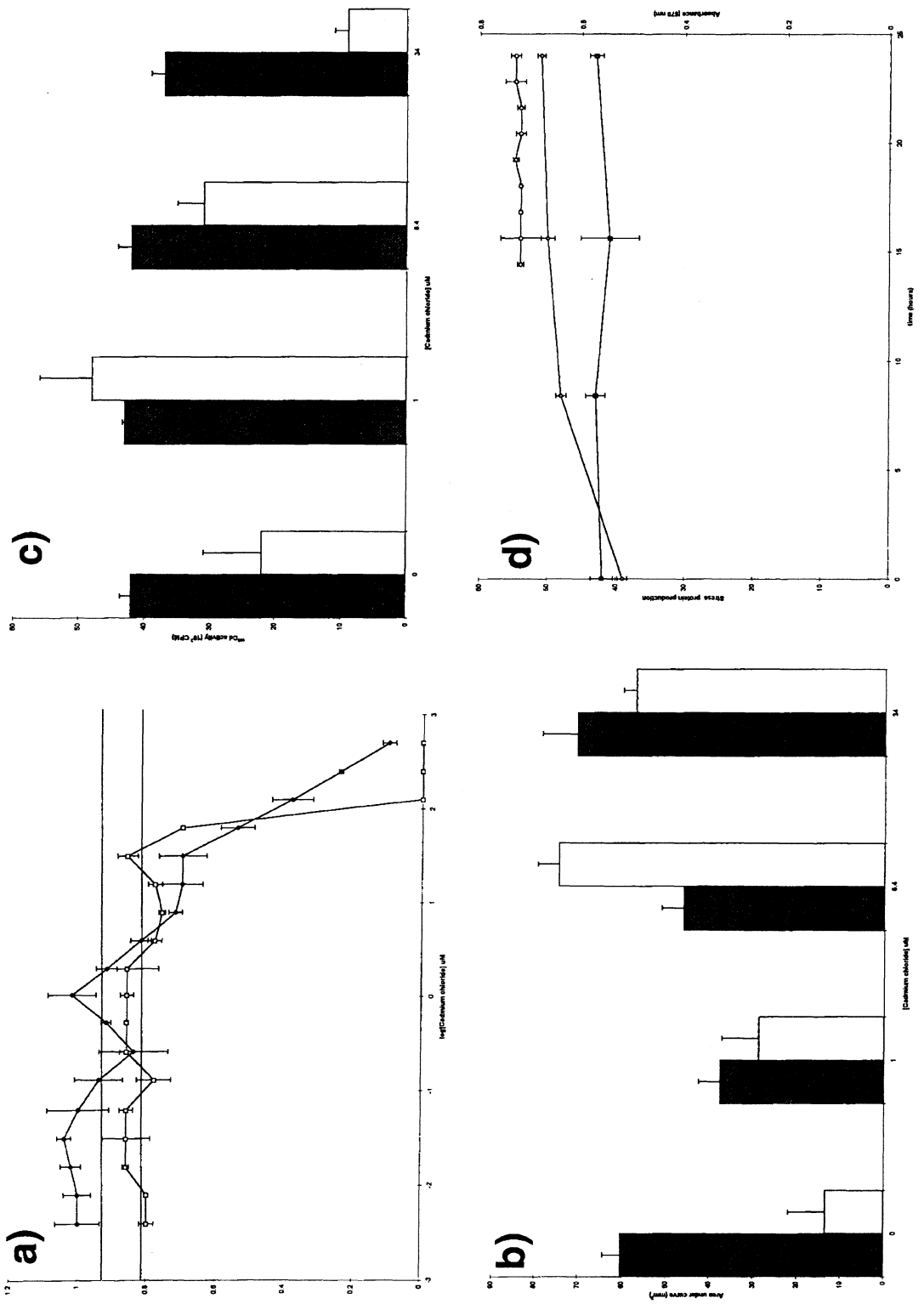


Figure 3 (a) A comparison of the cytotoxicity curves of CRM and normal cells exposed to $CdCl_2$ for 24 h. (diamond) Normal cells, (square) CRM cells. Solid lines represent untreated controls for each curve. A comparison of Hsp 70 (b) and metallothionein (c) levels in normal (white bar) and CRM cells (shaded bar) exposed to no cadmium chloride as well as hormetic (1 μM), plateau (8.4 μM) and LC_{50} (34 μM) concentrations of cadmium chloride for 24 h. (d) Time/activity assay and corresponding stress protein levels for CRM cells exposed to 1 μM cadmium chloride over 24 h. (diamond) Hsp 70 (peak area mm^2), (shaded square) metallothionein (^{109}Cd activity, 10^3 cpm), (circle) absorbance (570 nm).

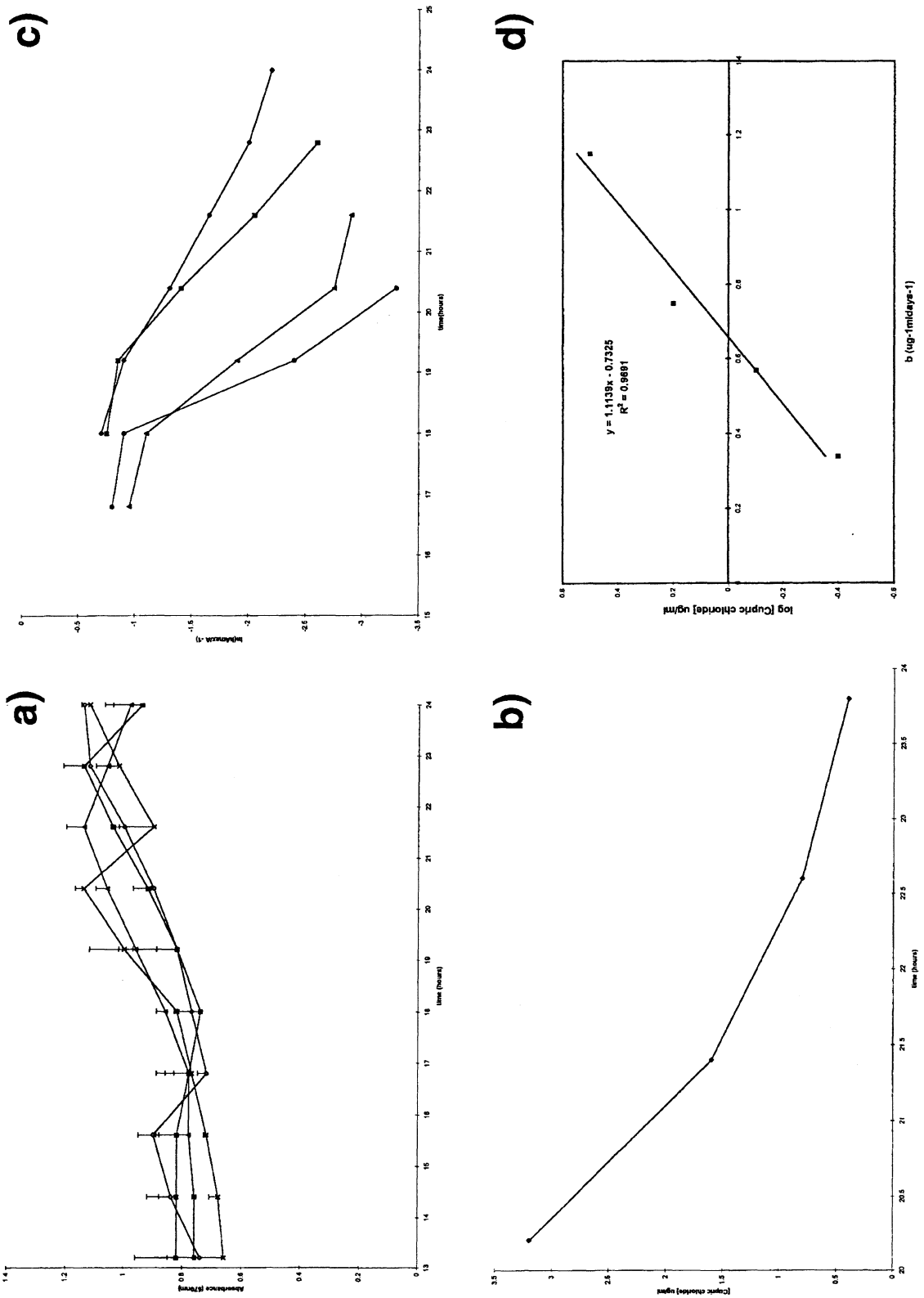


Figure 4 (a) Time/activity assay for cells exposed to cupric chloride. (diamond) 3 μM, (square) 6 μM, (triangle) 12 μM, (cross) 24 μM $CuCl_2$. (b) Metal concentration vs. time (t_{max}) (hours) for cells exposed to $CuCl_2$. (c) $\ln(hA_{max}/A-1)$ vs. time, (diamond) 3 μM, (square) 6 μM, (triangle) 12 μM, (circle) 24 μM $CuCl_2$. (d) $\log[C]$ vs. b for cells exposed to $CuCl_2$.

exposure time, we carried out time/activity studies for mercuric chloride (not shown) and cupric chloride.

We found that cells exposed to 3 μM cupric chloride exhibited peak hormetic activity at 23.8 h. At 6 μM, this peak activity regressed to 22.6 h and at 12 μM, hormetic activity regressed further to 21.4 h. At 24 μM cupric chloride, hormetic activity occurred at 20.2 h and then peaked again at 23.8 h, clearly indicating the dependence of hormetic onset time on concentration (Figure 4a). We plotted these concentrations against the time at which peak activity was observed and obtained an exponential curve (Figure 4b) which indicated that for a narrow concentration range, metal concentration (C) and the onset time of maximum activity (t_{max}) were exponentially related such that:

$$t_{max} := (-\ln C/C_0)/k \quad (1)$$

where k is a rate constant and C_0 is a theoretically large concentration at which the onset of hormetic activity would occur instantaneously (t=0). This suggested that a critical concentration of toxicant was required within cells to elicit increased activity and that the time taken to reach this critical concentration was dependent on the concentration of toxicant to which the cells were exposed, in a first-order manner.

The form of the curves in Figure 4a also suggested that at fixed metal concentrations, cellular activity tended to a maximum in a sigmoidal fashion with curves increasing in steepness as metal concentration increased. This suggested that activity peaks could be described by the sigmoidal equation:

$$A(t) := hA_{max}/(1 + e^{a-bt}) \quad (2)$$

where activity A tends over time t to a theoretical maximum A_{max} and where steepness or order of the curve is described by b, while a is a constant, dependent on control or 100% cellular activity. As it was not a certainty that the change in activity with time remained sigmoidal close to A_{max}, the constant h was included such that activity could then be said to tend over time to hA_{max} where we believe h tends to 1.

In order to determine whether the change in activity with time could be described in this manner, Equation 2 was rearranged to yield the straight line equation:

$$\ln(hA_{max}/A - 1) = -bt + a \quad (3)$$

and $\ln(hA_{max}/A-1)$ was plotted against time values for each time activity curve in Figure 4b using time intervals in which curves displayed sigmoidal characteristics. An average A_{max} value of 1.16 was used while h was assumed to be 1.

Plotting the data in this manner yielded curves analogous to hill plots.[7] where graphs consisted of linear central portions of slope b with deviations from linearity at each end (Figure 4c), clearly indicating that the change in activity with time was indeed sigmoidal within the chosen time intervals.

Since it was also apparent from activity/time plots that curve steepness increased with increasing metal concentration (Figure 4c), the manner in which b was related to concentration was investigated. A plot of the log of cupric chloride concentrations used in Figure 4a vs. the b values obtained from each corresponding curve exhibited a linear relationship (Figure 4d) and thus could be described by:

$$\log C := mb + \log C_{b_0} \quad (4)$$

where m is the slope of Equation 4 and C_{b_0} is a theoretically small concentration at which b is zero. Thus, it was now possible to substitute Equation 4 into Equation 2 yielding:

$$A(t) = hA_{max}/(1 + e^{a-[t\log(C/C_{b_0})]/m}) \quad (5)$$

which described the change in activity with time, where steepness of the slope b is expressed in terms of toxicant concentration.

From the above findings culminating in Equation 5, we were therefore able to describe hormetic activity evident in Figure 1a as composite curves where each log concentration value could be seen to represent a sigmoidal curve of specific order b, tending to a maximum activity which would be reached at a specific t_{max} value, itself dependent in an exponential manner on toxicant concentration (Equation 1).

Discussion

In this study, we have found that McCoy cells exposed to low concentrations of heavy metal chlorides exhibit significant increases in cellular activity and this activity, previously termed hormesis, coincides with the increased production of Hsp 70 and metallothionein.

Hsp 70 has been shown to be an essential component of the highly conserved and rapid molecular response displayed by cells when exposed to adverse environmental conditions.[8,9] In addition, metallothioneins are cysteine-rich, low-molecular-weight proteins (6 kDa) that have been found to be strongly induced in cells exposed to heavy metals and are thus believed to play an important role in the protection of cells against the toxic effects of metals and reactive oxygen species.[10,11]

Therefore, the increased levels of Hsp 70 and metallothionein in cells exhibiting hormetic activity,

as well as the high constitutive expression of these proteins coupled to high constitutive activity in CRM cells, strongly suggest that hormesis is a stress response to low but harmful concentrations of toxic agents.

In addition, kinetic studies have also revealed that the magnitude of the hormetic response is proportional to toxicant concentration, further suggesting that this phenomenon is a specific cellular response to a build-up of critical levels of metals within cells.

This, therefore, implies that the sigmoidal curves of different toxicants, which exhibit different steepness within the same concentration range, could be expected to have distinct *m* values dependent on the intrinsic qualities of the toxicant or toxicant/ligand complex which determine its ability to enter the cell and interact with cellular components. These values may thus have potential use as indicators of specific toxicants or toxicant combinations in unknown samples which induce hormetic peaks.

Although the above findings show that hormesis is an indicator of stress, the precise mechanisms responsible for its initiation and regulation remain unclear. But since the magnitude of the induced stress response was shown to be proportional to the order of the hormetic slope and inversely proportional to the onset of hormesis, with increasing toxicant concentrations, it is possible that this phenomenon is a form of energy compensation initiated by cells exposed to extreme environmental conditions which require the production of increased levels of stress proteins over a short period of time and where the degree of stress protein production is proportional to the magnitude of the stress to which the cells are exposed.

It has previously been shown that cells exposed to a variety of stresses exhibit a significant depletion in ATP levels.[12,13] Under these conditions, hormesis may be a rapid burst of energy production which serves to restore energy homeostasis in the cell. This hypothesis is supported by the findings evident in Figure 2b where hormesis was found to occur at maximum stress protein production, rather than occurring incrementally with increasing stress protein levels. This implies that the production of stress proteins and a subsequent drop in ATP levels are prerequisites for the induction of hormesis.

In addition, the sigmoidal kinetics of hormetic activity, which is often indicative of the amplified responses observed for cascade systems and the effects of allosteric modifiers on biochemical pathways, further suggests that this increased activity may be coupled to or regulated by specific stress-induced pathways. One possibility is the stress-activated protein kinase (SAPK)/c-Jun amino-terminal kinase (JNK) signalling pathway which has been found to be potently induced by protein synthesis inhibitors, heat shock and UV radiation, and is also believed to coordinate apoptosis in cells exposed to severe environmental stress.[14,15]

Therefore, an investigation of the relationship between hormesis and stress-induced signalling pathways and the induction of hormesis by a range of non-metal toxicants would be some of the aims of future research on this phenomenon.

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