

REPORT DOCUMENTATION PAGE

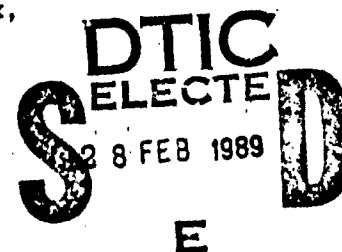
Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified			1b. RESTRICTIVE MARKINGS DTIC FILE COPY		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution unlimited - approved for public release		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE					
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION U.S. Army Medical Research Institute of Infectious Diseases		6b. OFFICE SYMBOL (If applicable) SGRD-UIS	7a. NAME OF MONITORING ORGANIZATION U.S. Army Medical Research and Development Command		
6c. ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, MD 21701-5011			7b. ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, MD 21701-5012		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION		8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DTIC SELECTED 28 FEB 1989		
8c. ADDRESS (City, State, and ZIP Code)			10. SOURCE OF FUNDING NUMBERS		
			PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO. WORK UNIT ACCESSION NO. S E
11. TITLE (Include Security Classification) Effect of Anti-Hepatotoxic Agents Against Microcystin-LR Toxicity in Cultured Rat Hepatocytes					
12. PERSONAL AUTHOR(S) Kay A. Mereish and Rikki Solow					
13a. TYPE OF REPORT Interim		13b. TIME COVERED FROM _____ TO _____		14. DATE OF REPORT (Year, Month, Day) 10 Jan 89	
15. PAGE COUNT 22					
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	microcystin-LR, hepatocytes, anti-hepatotoxic, dithioerythritol, silymarin		
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION		
22a. NAME OF RESPONSIBLE INDIVIDUAL			22b. TELEPHONE (Include Area Code)		22c. OFFICE SYMBOL

EFFECT OF ANTI-HEPATOTOXIC AGENTS AGAINST MICROCYSTIN-LR
TOXICITY IN CULTURED RAT HEPATOCYTES

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RUNNING TITLE: ANTI-HEPATOTOXINS AGAINST MICROCYSTIN-LR

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Effect of Anti-hepatotoxic Agents Against Microcystin-LR Toxicity in Cultured Rat Hepatocytes. MEREISH, K.A. AND SOLOW, R. (1989). *Toxicol. Appl.*

Pharmacol., - . Primary cultures of adult rat hepatocytes were used to investigate the effects of two putative therapeutic agents, dithioerythritol and silymarin on microcystin-LR-induced hepatotoxicity. Cell injury was assessed by (1) the extent of cellular [14 C]adenine nucleotides and lactate dehydrogenase (LDH) release into the media and (2) the extent of hepatocyte detachment from monolayers. Microcystin-LR (1 μ M) induced a significant release of both [14 C] nucleotides and LDH from hepatocytes as well as significant detachment of cells from monolayers. Although both dithioerythritol and silymarin reduced the amount of marker release and cell detachment from microcystin-LR-treated wells, silymarin provided significantly greater protection than dithioerythritol at one tenth the concentration. Furthermore, silymarin and dithioerythritol treatment prevented morphological deformations and detachment of cells.

Cyanobacteria, *Microcystis aeruginosa*, synthesize several related, small-molecular-weight, cyclic polypeptides (Bishop et al., 1959) which possess potent hepatotoxic activity in many species, including humans (Gorham 1964; Gorham and Carmichael, 1979; Falconer et al., 1983; Jackson et al., 1986). One of the most potent of these peptides, microcystin-LR, is a 7-amino acid ring structure where L and R designate the two variant amino acids, leucine and arginine, respectively (Botes et al., 1984). Administration of lethal doses of microcystin-LR to laboratory rodents rapidly induces severe liver hemorrhage which is associated with centrilobular hepatocyte necrosis (Schwimmer and Schwimmer, 1964; Falconer et al., 1981).

Microcystin-LR not only induces the rapid onset of liver damage in rodents *in vivo* (Slatkin et al., 1983; Falconer et al., 1981; Runnegar and Falconer, 1981; Theiss 1984; Theiss and Carmichael, 1986), but also induces necrosis of cultured rat hepatocytes after several hours of incubation with the toxin (Foxall and Sasner, 1981). These observations have led to the suggestion that microcystin-LR may cause the destruction of the sinusoidal endothelial lining and/or disintegration of hepatocyte cell membranes (Falconer et al., 1981).

Microcystin-LR-induced, *in vitro* cytotoxicity can be separated into early and late events. The early events that occur within seconds to minutes after hepatocytes are exposed to the toxin are characterized by morphological deformation of cells (blebbing) (Runnegar et al., 1981), rapid rise in intracellular calcium, increased phosphorylase-a activity, depletion of glutathione (Runnegar et al., 1987; Falconer and Runnegar, 1987), and release of arachidonic acid metabolites (Naseem et al., 1986). These early events are not associated with cell toxicity (as measured by trypan-blue exclusion) and do not depend on the presence of external calcium (Falconer and Runnegar,

1987). The late events, however, occurring over several hours after the exposure of hepatocytes to microcystin-LR, are characterized by the leakage of adenine nucleotides and cytosolic enzymes, followed by loss of cell viability (Mereish et al., 1989, in preparation). The mechanism by which microcystin-LR induces hepatotoxicity is not known. Several hypotheses exist concerning the interaction of microcystin-LR with hepatocytes. They included whether the toxin is: transported into the cell via the bile acid transporters of the cell membrane (Thompson et al., 1988), metabolically activated (Adams et al., 1985; Stohs, personal communication, 1988) with subsequent covalent binding of metabolites to cellular macromolecules (as was shown for cyclosporine) (Nagelkerke et al., 1987), or through the release of soluble inflammatory mediators (Naseem et al., 1988).

Despite the lack of knowledge about the mechanism of action of microcystin-LR, we investigated the effect of dithioerythritol (DTE) and silymarin (SM) on microcystin-LR-induced toxicity of cultured rat hepatocytes. DTE, which is also known as Cleland's reagent, is an excellent reagent for maintaining thiol (-SH) groups in the reduced state (Cleland, 1964) and is frequently used as a protective agent *in vitro* against hepatotoxins that produce oxygen-free-radical-induced, oxidative stress in cultured hepatocytes (Nicotera et al., 1984; Bellomo et al., 1987). SM, a 3-arylflavonone isolated from the fruit of *Silybum marianum*, has been shown to have antihepatotoxic effects *in vivo* (Hahn et al., 1968) and *in vitro* (Wagner, 1986; Hikino et al., 1984).

In order to determine if DTE and SM have a protective effect against microcystin-LR induced toxicosis, cultured rat hepatocytes were pretreated with these agents and then exposed to microcystin-LR. Microscopy, the release of both [¹⁴C]adenine nucleotides and LDH from cultured hepatocytes (Shirhatti

and Krishna, 1985), as well as detachment of hepatocytes from culture, were used as indices of cell injury.

MATERIAL AND METHODS

Materials. The following materials were obtained commercially from the indicated sources: SM (Aldrich Chemical Co. Inc., Milwaukee, WI); [¹⁴C]adenine (50 mCi/mmol) (New England Nuclear Corp., Boston, MA); tissue culture medium and fetal bovine serum albumin (GIBCO, Grand Island, N.Y.); tissue culture ware (Becton-Dickinson Labware, Lincoln park, NJ); and rat tail collagen, collagenase type IV, 5'-adenosine monophosphate (AMP), 5'-adenosine diphosphate (ADP), 5'-adenosine triphosphate (ATP), 5'-inosine monophosphate (IMP), adenosine, adenine and DTE (Sigma, St. Louis, MO). Fluorescent poly(ethylene)imine cellulose plates (PEI) were obtained from EM Science.

Livers from male FW.LEW, congenic, inbred rats (C. Anderson, USAMRIID, Fort Detrick, Frederick, MD), weighing between 250 - 300 g, were used for all experiments. Microcystin-LR (85-95% purity) was obtained from Dr. W. Carmichael, Wright State University, Dayton, Ohio.

Hepatocytes. Rat hepatocytes were isolated and cultured according to the methods of Elliget and Koland (1983). Viable hepatocytes were counted with a hemocytometer and using trypan blue in phosphate-buffered solution. Hepatocytes were suspended at 5×10^5 viable cells per ml in Leibovitch's (L15) medium containing 17% fetal calf serum (FCS) and were seeded on collagen-coated, 6-well plates by adding 1 ml of cell suspension per well. The cells were allowed to settle for 30 min at room temperature and then incubated at 37°C with 5% CO₂ and 90% humidity for an additional 2 hr. After incubation, the majority of the cells had attached to the bottom of the well and

established a monolayer. The non-attached cells were removed by aspiration and 2 ml of fresh culture medium was added to each well.

Labeling the nucleotide pool and measurement of drug-induced toxicity. After overnight incubation of the hepatocytes, culture medium from each well was replaced with 1 ml of L15 medium containing ^{14}C -adenine (0.2 μCi , 4 μM). Adenine nucleotide pool was labeled as described by Shirhatti and Krishna (1985). The labeled cells were then incubated for 30 min with 0.5 ml of L15 medium containing varying concentrations of DTE, SM, or medium as control. At the end of this incubation, another 0.5 ml of cultured medium was added to the cells, either with or without microcystin-LR. The cells were reincubated for additional 6 hr, after which cell supernatants were removed and centrifuged at 500 X g for 4 min in an Eppendorf centrifuge, model 5414. An aliquot (200 μl) of each supernatant was removed and counted for radioactivity in 10 ml of Hydroflour (National Diagnostic, Somerville, NJ) in a Beckman scintillation counter, model LS5800 (Beckman Inst. Co., Fullerton, CA). Another aliquot of the supernatant was removed and stored at -4°C for adenine nucleotides and LDH enzyme activity assay. The cells were lysed by the addition of 1 ml of 0.05% digitonin in phosphate buffer to each well. An aliquot of each cell lysate was removed in order to measure radioactivity, LDH, and protein content. Protein levels were determined using Pierce protein reagent (Pierce, Rockford, IL) and bovine serum albumin as the standard. LDH was assayed with sodium lactate as substrate and NAD as the cofactor; the rate of formation of NADH was monitored at 340 nm using Cobas Bio (Roche Analytical Inst., Nutley, NJ).

[¹⁴C]Adenine nucleotides (AMP, ADP, ATP, IMP), adenine, and adenosine were determined by thin-layer chromatography (TLC). Aliquots of cell lysate and supernatant samples, along with standards, were chromatographed on PEI-cellulose plates. The plates were developed as described by Bochner and Ames (1982). The regions corresponding to those of the chromatographed standards were scraped from the plate and counted for radioactivity. Leakage of ¹⁴C-labeled nucleotides and cytosolic LDH from hepatocytes was determined for control, SM-, and DTE-treated cells, with and without the presence of microcystin-LR.

Cell Viability. Hepatocyte viability was assessed by light microscopy using a Nikon Diphot inverted phase contrast microscope. Photographs were taken with a Nikon FE camera and Tungston 50, 35-mm, color slide film. Many hepatotoxins, including microcystin-LR, reduce cell viability as shown by detachment of hepatocytes from the surface of culture plates (Shiratti and Krishna, 1985). Consequently, in toxin-treated cells, the number of attached cells will decrease with time, which will be reflected by a decrease in protein amounts as compared to control wells. Therefore, we measured the amount of protein associated with attached control, microcystin-LR, SM- and DTE-treated cells after 6 hr incubation. The amount of protein from attached cells per well was used as an additional index of cell viability.

RESULTS

Treatment of hepatocytes with DTE (0.63 - 5 mM) significantly reduced the amount of both ¹⁴C-adenine nucleotide and LDH (Fig. 1) released from microcystin-LR-exposed cells. Similarly, SM treatment (25 to 200 μ M)

significantly reduced the release of both markers (Fig. 2). Maximum protection of hepatocytes against microcystin-LR toxicity was achieved with 200 μ M SM and with 2.5 mM of DTE.

The Rf values for AMP, ADP, ATP, IMP and adenosine were 0.68, 0.34, 0.1, 0.58, and 0.54, respectively. Due to the poor resolution in separating IMP from adenosine, the bands corresponding to both compounds was as band and reported as IMP.

Approximately 95% of the [14 C]adenine taken up by control hepatocytes was incorporated into the total cellular adenine nucleotide pool (data not shown). The majority of [14 C]nucleotides released into the medium from control cells after 6 hr of incubation was deaminated AMP (IMP) and/or adenosine. Although microcystin-LR induced a significant overall loss of the cellular adenine nucleotide pool, it did not change its distribution (IMP and AMP 89%; ADP, 8%; ATP, 0.5%; adenine, 1.6%).

Microscopy of control cells revealed that the majority of cells remained rectangular, mono and binucleated (Fig 3a) and attached to the bottom of their culture plates for the duration of the incubation period. Microcystin-LR treated cells, however, became rounded, deformed (blebbed) (Fig 3b) and detached from the culture plates. This was reflected by low protein concentrations associated with microcystin-LR- treated cells compared to controls. Treatment with DTE or SM prevented hepatocyte deformation (Fig. 3c,d) and detachment (Fig. 4) from plates after exposure to microcystin-LR.

DISCUSSION

Although both DTE and SM stabilized hepatocytes with respect to the release of ^{14}C -adenine nucleotides and LDH, SM provided protection against microcystin-LR-induced toxicity at concentrations lower than DTE. Since DTE and SM protected hepatocytes against other hepatotoxins, whose mechanisms of action are better understood, the information gleaned from these studies may therefore provide insight into the possible protective mechanisms of both agents against microcystin-LR induced toxicosis.

Toxins such as acetaminophen, N-acetyl-p-benzoquinone, bromobenzene, dicoumarol, menadione, and t-butyl hydroperoxide injure hepatocytes by inducing oxidative stress by generating free radicals (Thor *et al.*, 1982). The generation of free radicals leads to the depletion of cellular reduced glutathione; covalent binding of free radical metabolites to cellular macromolecules; and oxidation of protein thiol groups, leading to the subsequent inhibition of the Ca^{++} translocases located in the plasma membrane, endoplasmic reticulum, and mitochondrial inner membrane. These alterations are eventually translated into membrane peroxidation, phospholipase A2 activation, disturbance of Ca^{++} homeostasis, deformation of the cell surface (blebbing), and the loss of cell viability (Dimonte *et al.*, 1984; Moore *et al.*, 1985). Although it has been shown that toxin-induced glutathione depletion, covalent binding to macromolecules, and lipid peroxidation occur prior to cell death, these events do not always correlate with the loss of cell viability (Siegers *et al.*, 1977; Stacey and Klaarsen, 1981).

The inhibition of the $\text{Ca}^{++}\text{Mg}^{++}\text{ATPase}$, especially of the endoplasmic reticulum, and the consequent sustained disturbance of intracellular Ca^{++} homeostasis have been shown to correlate well with the loss of cell viability after carbon tetrachloride and thioacetamide intoxication (Younes *et al.*

1983). The activity of these Ca^{++} translocases has been shown to depend critically on the reduced state of their thio groups (Bellomo et al., 1983; Moore et al., 1975)

Addition of DTE to the culture medium of toxin-treated hepatocytes reversed the early morphological changes and protected them against the loss of viability associated with free radical-induced oxidative stress (Starke et al., 1986; Maridonneau-Parimi et al., 1986). Most investigators believe that DTE protects cells not by directly interacting with either the toxin nor cellular components, but by reducing oxidized thiol groups associated with critical proteins, i.e., Ca^{++} translocases (Tee et al., 1986).

Although flavonoids, in general, have pleiotrophic effects on mammalian cells (Havsteen, 1983; Laychock, 1986), SM (and related compounds) has been shown to inhibit lipxygenase specifically and therefore leukotriene synthesis (Baumann et al., 1980); scavenge and neutralize free radicals generated during oxidative stress (Vengerovskii et al., 1987; Fraga et al., 1987; Valenzuela et al., 1986; Valenzuela and Guerra, 1986); and increase hepatocyte rRNA, ribosomal and protein synthesis *in vivo* and *in vitro* (Sonnenblicher and Zettl, 1986).

Microcystin-LR may produce hepatotoxicity by inducing a series of events that eventually overwhelms the cell's capacity to defend or repair itself. The early events associated with microcystin-LR-induced hepatotoxicity (blebbing, increase of intracellular Ca^{++} , increase of phosphorolase-a activity, glutathione depletion, and arachidonic acid release) are strikingly similar to the events seen with oxidative stress resulted from other toxins. These early events suggest that microcystin-LR may generate a free radical(s) and therefore induce oxidative stress with eventual disruption of membrane integrity, oxidation of thiol groups of key cellular proteins, and disturbance

of Ca^{++} homeostasis (Jewell et al., 1982).

It is possible that microcystin-LR inhibits the activity of one or all of the Ca^{++} -translocating pumps by binding to proteins directly and/or oxidizing their thiol groups. This possible, irreversible inhibition of the calcium pumps, followed by a sustained disruption of Ca^{++} homeostasis, may take place during the late period (2-3 hr) observed in our laboratory and lead to the release of [^{14}C] adenine nucleotides and LDH and finally the loss of cell viability. The effect of microcystin-LR on hepatocytes SH-proteins is currently under investigation in our laboratories.

SM may be superior to DTE against microcystin-LR-induced hepatotoxicity for several reasons. The protective effect of DTE exists only vis-a-vis its ability to reduce protein or glutathione thiol groups. This effect depends on having an adequate concentration of DTE available during the incubation period. SM, however exerts three effects, which could allow the cell to recover more effectively from the initial, reversible, toxin-induced effects. SM inhibits the synthesis and release of leukotrienes, which have been shown to mediate hepatocyte damage induced by endotoxin and N-acetylgalactosamine toxicosis (Hagmann et al., 1985; Keppler et al., 1985). Furthermore, SM decreases the concentration of free radicals, especially superoxide and hydroxyl radicals (Valenzuela and Guerra, 1986; Valenzuela et al., 1986), and increases overall protein synthesis (Sonnenbicher and Zetl, 1986). In summary, we conclude that SM and DTE may protect against microcystin-LR induced-hepatotoxicity by preventing the synthesis and release of arachidonic acid metabolites, scavaging possible free radicals generated during microcystin-LR toxicosis, and preserving the reduced state of thiol groups of critical cellular proteins. Testing the efficacy of SM in microcystin-LR toxicosis in whole animal models is in progress.

ACKNOWLEDGEMENTS

We thank Karen Bostian, Lori Weaver, and Rosa Searles for providing technical support, Drs. Donald Creasia and John Hewetson for revising the manuscript, and Jackie Chaco-Farley for typing.

FIG. 1. Effect of 1 μ M of microcystin-LR on [14 C]adenine nucleotide (●—●) and LDH (○—○) release from cultured rat hepatocytes treated with DTE. Cells were incubated with [14 C]-adenine for 1 hr. The hepatocytes were then washed and reincubated in 0.5 ml medium containing selected concentrations of DTE for 30 min. An additional 0.5 ml medium alone or medium with 1 μ M microcystin-LR was added to each well and cells were then incubated for 6 hr. After incubation, cell supernatants and cellular [14 C]nucleotides and LDH were determined as described in the text. The results are presented as the percent of marker release. Each point represents the mean of three determinations $\pm$ SD.

FIG. 2. Effect of 1 μ M microcystin-LR on [14 C]adenine nucleotides (●—●) and LDH (○—○) release from cultured rat hepatocytes treated with SM. Cells were treated as described in Fig. 1, except that SM was used instead of DTE. The results are presented as the percent of marker released. Each point represents the mean of three determinations $\pm$ SD.

FIG. 3. Phase contrast (x 80) of 6-hr cultures of (a) control hepatocytes, (b) hepatocytes treated with 1 μ M microcystin-LR, (c) hepatocytes treated with 5 mM of DTE and 1 μ M of microcystin-LR, (d) hepatocytes treated with 0.2 mM of SM, and 1 μ M of microcystin-LR. Cell blebbing is indicated by an arrow.

FIG. 4. Effect of DTE (●—●) and SM (■—■) on cell attachment. Cells were treated as described in Fig. 1 except that protein was determined for attached and detached cells as described in the text. The results are presented as the percent of attached cellular protein to total protein per well. Each point represents the mean of three determinations $\pm$ SD.

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