0026-895X/99/061324-05$3.00/0

MOLECULAR PHARMACOLOGY, **56:**1324–1328 (1999).

Protective Effects of Triterpene Compounds Against the Cytotoxicity of Cadmium in HepG2 Cells

NOBUHIKO MIURA, YOKO MATSUMOTO, SHINICHI MIYAIRI, SHOJI NISHIYAMA, and AKIRA NAGANUMA

Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (N.M., Y.M., S.M., A.N.); and Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Yokohama, Japan (S.N.)

Received July 12, 1999; accepted September 8, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

The effects of triterpene compounds on cadmium toxicity were investigated in HepG2 cells. Ten triterpene compounds were examined, namely, betulin, soyasapogenol A, soyasapogenol B, ursolic acid, uvaol, oleanolic acid, friedelin, glycyrrhizin, 18α-glycyrrhetinic acid, and 18β-glycyrrhetinic acid, and betulin, soyasapogenol A, and uvaol were found to reduce the toxicity of $CdCl_2$. In particular, betulin almost completely abolished the cytotoxicity of $CdCl_2$ at concentrations as low as 0.1 μg/ml. The effects of betulin were particularly apparent when added to the culture medium before the addition of $CdCl_2$. Moreover, when HepG2 cells were incubated with betulin and then incubated in fresh betulin-free medium before the addition of $CdCl_2$, the toxic effects of cadmium were reduced. Betulin had no significant effect on the intracellular accumulation of cadmium, nor did it bind to cadmium, at least not in a test tube. When HepG2 cells were treated first with cycloheximide or actinomycin D, the subsequent protective effect of betulin against cadmium toxicity was significantly reduced, suggesting that betulin might protect cells against cadmium toxicity by inducing the synthesis of a certain protein or proteins. The synthesis of metallothionein, a protein that is known to reduce the toxicity of heavy metals, was not induced by betulin. However, using the differential display method, we confirmed that betulin promoted the expression of several genes. Our findings suggest that betulin might reduce cadmium toxicity by promoting the synthesis of certain proteins that protect cells against the toxic effects of cadmium.

When cadmium is administered as $CdCl_2$ to animals, most of the cadmium accumulates initially in the liver (Webb, 1979). Accordingly, the acute toxic effects of cadmium are observed mainly in the liver (Webb, 1979). It has been reported that the hepatotoxicity of cadmium can be reduced by plant triterpenes, such as oleanolic acid (Liu et al., 1993b, 1994b), ursolic acid (Liu et al., 1994b), α-hederin (Liu et al., 1993a, 1994b), and glycyrrhizin (Shaikh et al., 1999). Triterpenes are aliphatic polycyclic compounds based on a skeleton with 30 carbon atoms, and they are synthesized from squalene as the precursor (Mahato et al., 1988). Triterpenes are present in all parts of plants, such as the roots, pollen, fruits, and seeds (Mahato et al., 1988). Anti-inflammatory effects, antitumor effects, protective effects on the liver, and immunoregulatory effects have all been reported as biological effects of triterpenes (Ma et al., 1986; Tokuda et al., 1986; Price et al., 1987; Mahato et al., 1988). In particular, the protective effects on the liver are of major significance, and some triterpenes have been shown to reduce the hepatotoxicity of carbon tetrachloride and acetaminophen (Hikino, 1985; Liu et al., 1994a,b). However, there is as yet no full understanding of the mechanism of protection of the liver by triterpenes. Therefore, in the present study, to obtain information about the mechanisms whereby triterpenes reduce cadmium toxicity, we investigated the effects of triterpenes on the cytotoxicity of $CdCl_2$ using a line of cells derived from human liver, namely, HepG2 cells. We found that betulin, a triterpene that is present at high levels in the bark of white birch (*Betula alba L.*), very considerably reduced the cytotoxicity of cadmium. We also obtained evidence that suggests that the effect of betulin results from the induction of the synthesis of certain intracellular proteins that is caused by the enhanced expression of certain, as yet unidentified, genes.

Materials and Methods

Chemicals and Cells. Triterpene compounds were provided by Meiji Seika Kaisha Ltd. (Tokyo, Japan) and were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) before use. HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with kanamycin sulfate (60 μg/ml; Life Technologies Inc., Rockville, MD), $NaHCO_3$ (0.1%; Nacalai Tesque), L-glutamine (316 mg/ml; Nacalai Tesque), and FBS (10%; JRH Biosciences, Lenexa, KS) in an atmosphere of 5% CO_2 in air at 37°C. Alamar Blue was obtained from Alamar Biosciences Inc. (Sacramento, CA). All other reagents that were used were of the highest grade available commercially.

ABBREVIATIONS: DMSO, dimethyl sulfoxide; MT, metallothionein.

Cytotoxicity of Cadmium. HepG2 cells were seeded onto 96-well microplates at a cell density of 1×10^4 cells/well, and the solutions of individual triterpenes were added 24 h later. Cadmium chloride was added 24 h after the addition of individual triterpenes, and the cells were cultured for an additional 24 h. For estimation of the rate of cell survival, Alamar Blue was added to the medium, and absorbance was monitored at 570 and 620 nm after culture for 3 h as described. To control for the effects of DMSO, we adjusted the final concentration of DMSO in the medium to 1% (v/v) in all cultures, including the controls.

Determination of Accumulation of Cadmium. HepG2 cells were seeded onto 6-well plates at a cell density of 4×10^5/well. After culture for 24 h, betulin was added, and the cells were cultured for an additional 24 h. Each culture was supplemented with $[^{109}Cd]CdCl_2$ (20 μCi; NEN Life Science Products, Boston, MA) to give a final concentration of 20 μM, and then cells were incubated for 3 or 24 h. The medium was removed, and the cells were rinsed twice with PBS(−) that contained 1 mM EDTA to remove any cadmium that had bound nonspecifically to cell surfaces. Then, the radioactivity of ^{109}Cd that had accumulated in the cells was determined with a gamma counter.

Effects of Inhibitors of Protein and RNA Synthesis. HepG2 cells were seeded onto 96-well microplates at a cell density of 1×10^4 cells/well. After culture for 18 h, the inhibitor of protein synthesis cycloheximide (2 μg/ml) or the inhibitor of RNA synthesis actinomycin D (0.05 μg/ml) was added. Six hours later, betulin (0.5 μg/ml) was added to each culture. Because cycloheximide and actinomycin D have cytotoxic effects when cells are cultured with these compounds for a prolonged period, the medium was replaced with fresh drug-free medium 4 h after the addition of betulin. Cadmium chloride was added 20 h later, and the rate of cell survival was determined 24 h after the addition of $CdCl_2$ with Alamar Blue, as described above.

Quantification of Metallothionein (MT). HepG2 cells were seeded onto 6-well plates at a cell density of 4×10^5/well. After

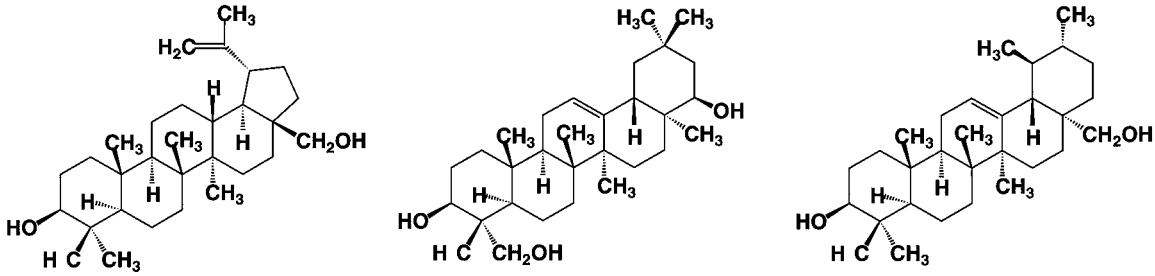

Fig. 1. Structures and molecular weights of betulin, soyasapogenol B, and uvaol.

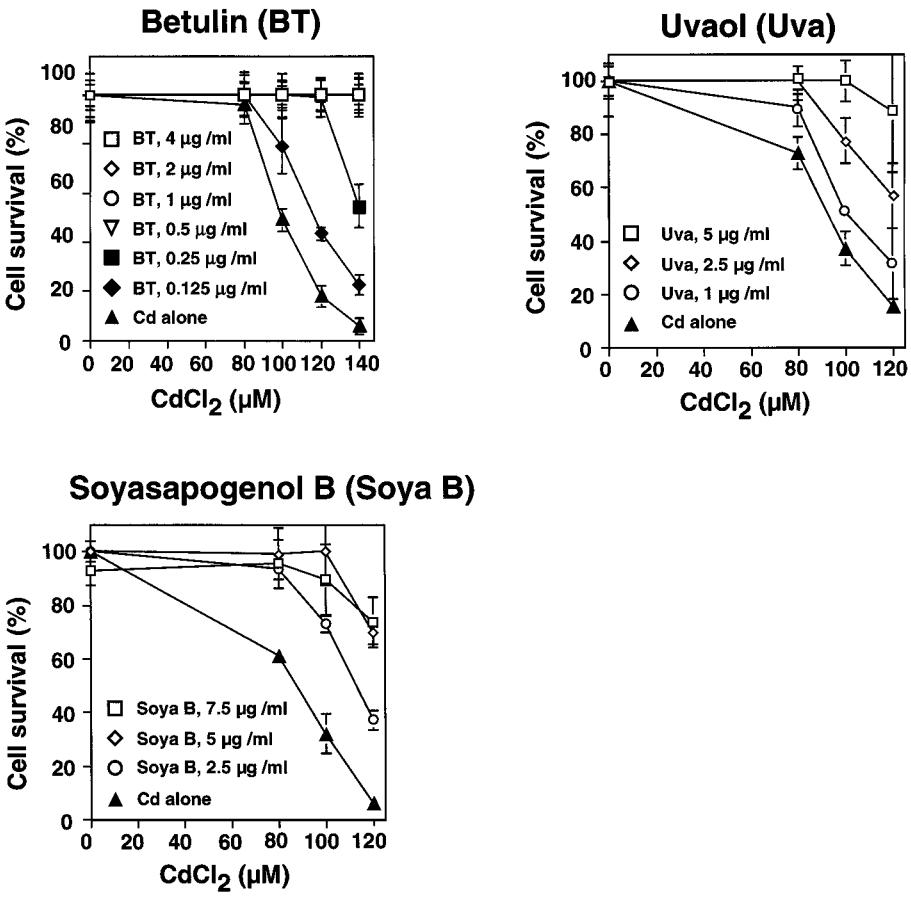

Fig. 2. Effects of three triterpenes on the toxicity of $CdCl_2$ in HepG2 cells. HepG2 cells were treated with betulin, uvaol, or soyasapogenol B for 24 h, and then $CdCl_2$ (Cd) was added to the culture medium at indicated concentrations. After incubation for 24 h, cell survival was determined with Alamar Blue as described in the text. Each point represents the mean value of results from three cultures with S.D. (bars).

culture for 24 h, betulin (0.5 μg/ml) or a strong inducer of the synthesis of MT, $ZnCl_2$ (150 μM; as a positive control), was added, and cells were cultured for an additional 24 h. The cells were rinsed twice with PBS(−), removed from the plates, and collected by centrifugation at 1500 rpm for 5 min. The amounts of MT in the pelleted cells were determined by HPLC with detection of fluorescence, as described by Miyairi et al. (1998).

Screening by Differential Display Method. HepG2 cells were seeded onto 10-cm dishes at a cell density of 3×10^6 cells/dish. After culture for 24 h, betulin was added at a final concentration of 4 μg/ml. Total RNA was extracted with Isogen (Nippon Gene, Tokyo, Japan) 4 or 8 h after the addition of betulin. Differential display (Liang and Pardee, 1992) was archived with RNA Image Kits 1 and 2 (GenHunter, Nashville, TN). Single-stranded cDNA was synthesized by reverse transcription from the total RNA (0.1 μg) using anchored oligo(dT) primers (H-T_{11}G, H-T_{11}A, and H-T_{11}C; GenHunter) and Moloney murine leukemia virus reverse transcriptase. The cDNAs were mixed with the reaction solution for PCR [Ampli *Taq* Gold (0.5 U; PE Applied Biosystems, Tokyo, Japan), 2.5 μM concentrations of dNTPs, and [α-^{32}P]dCTP (0.05 μCi; NEN Life Science Products) that contained one of the above-mentioned anchored oligo(dT) primers and arbitrary primers (arbitrary 13-mers, H-AP primers 1–16; GenHunter). After heating at 95°C for 10 min in a thermal cycler, the reaction mixture was subjected to 40 cycles of amplification that each consisted of incubation at 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s. Amplification was followed by one cycle of elongation at 72°C for 5 min. The products of polymerase chain reaction were fractionated by electrophoresis on a 6% polyacrylamide gel that contained urea. The gel was dried and exposed to radiographic film at −80°C for 15 to 25 h for autoradiography.

Results and Discussion

To our knowledge, the effects of triterpenes on cadmium toxicity have not previously been investigated in cultured cells. Therefore, we investigated the effects of 10 triterpenes (betulin, soyasapogenol A, soyasapogenol B, ursolic acid, uvaol, oleanolic acid, friedelin, glycyrrhizin, 18α-glycyrrhetinic acid, and 18β-glycyrrhetinic acid) on the cytotoxicity of $CdCl_2$ in HepG2 cells. Triterpenes were tested at concentration ranges at which each individual compound was not toxic to cells (rate of cell survival were 95% or higher). Triterpenes were added to the culture medium 24 h before the addition of $CdCl_2$. Under these conditions, betulin, soyasapogenol B, and uvaol (their structures are shown in Fig. 1) markedly reduced the toxicity of $CdCl_2$. Most HepG2 cells that had been treated with these compounds survived subsequent exposure to $CdCl_2$ at a concentrations close to the 100% lethal concentration (Fig. 2). By contrast, oleanolic acid, glycyrrhizin, or ursolic acid had no significant effects, even though they were reported previously to reduce the hepatotoxicity of cadmium (Liu et al., 1994a,b; Shaikh et al., 1999). Soyasapogenol A, friedelin, 18α-glycyrrhetinic acid, and 18β-glycyrrhetinic acid also had no protective effects. Liu et al. (1994a) reported that uvaol reduced cadmium-induced hepatopathy in mice, but there have been no reports, to our knowledge, that betulin and soyasapogenol B reduce cadmium toxicity. We found that betulin, in particular, very strongly reduced the cytotoxicity of $CdCl_2$ at concentrations as low as 0.5 μg/ml. There are no reports, again to our knowledge, of any compound that reduces the cytotoxicity of cadmium as effectively as betulin. A protective effect of betulin against cadmium toxicity was observed in NIH3T3 and P388 cells, as well as in HepG2 cells, but not in mouse L and colon 26 cells (data not shown), suggesting that the protective effect may be cell-type depen-

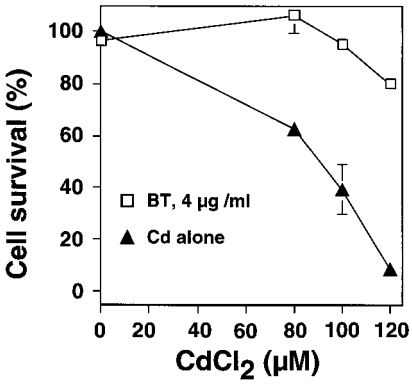

Fig. 4. Effects of prior treatment with betulin on the subsequent toxicity of $CdCl_2$ in HepG2 cells. HepG2 cells were treated with betulin (BT) for 2 h, from 24 to 22 h before the addition of $CdCl_2$ (Cd), and then cells were transferred to betulin-free medium. After a 22-h incubation in betulin-free medium, $CdCl_2$ was added at indicated concentrations, and incubation was continued for 24 h. Cell survival was determined with Alamar Blue. Each point represents the mean value of results from three cultures with S.D. (bars). The absence of a bar indicates that the S.D. falls within the symbol.

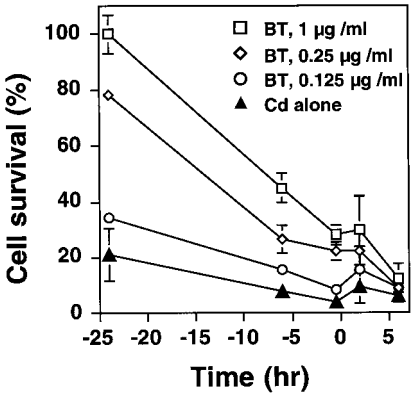

Fig. 3. Effects of the timing of treatment with betulin on the toxicity of $CdCl_2$ in HepG2 cells. Betulin (BT) was added to cultures of HepG2 cells 24, 6, and 0.5 h before and 2 and 6 h after the addition of $CdCl_2$ (Cd) at 120 μM. Cell survival was determined with Alamar Blue after an additional 24-h incubation. Each point represents the mean value of results from three cultures with S.D. (bars).

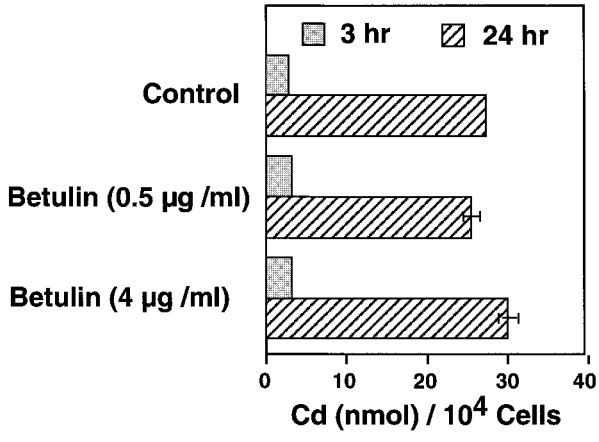

Fig. 5. Effects of betulin on the accumulation of cadmium in HepG2 cells. Betulin was added to cultures of HepG2 cells 24 h before the addition of ^{109}Cd-labeled $CdCl_2$ (20 μM). Cellular accumulation of ^{109}Cd was determined after a 3- or 24-h incubation with ^{109}Cd-labeled cadmium as described in the text. Results are mean of results from four cultures with S.D. values (bars).

dent. Betulin is a relatively abundant compound in the bark of white birch (*Betula alba L.*). Although betulin is known to have antimalarial (Steele et al., 1999), anti-human immunodeficiency virus (Sun et al., 1998a,b), and anti-inflammatory effects (Recio et al., 1995), the mechanisms of its actions have not yet been elucidated.

We also examined the effects of betulin on the cytotoxicity of zinc chloride, mercuric chloride, methylmercury chloride, and cisplatin, all of which are heavy-metal compounds, and of hydrogen peroxide, paraquat, and Adriamycin, which are inducers of oxidative stress. In no case did betulin have any significant effect on toxicity. Thus, betulin appeared specifically to reduce the toxicity of cadmium.

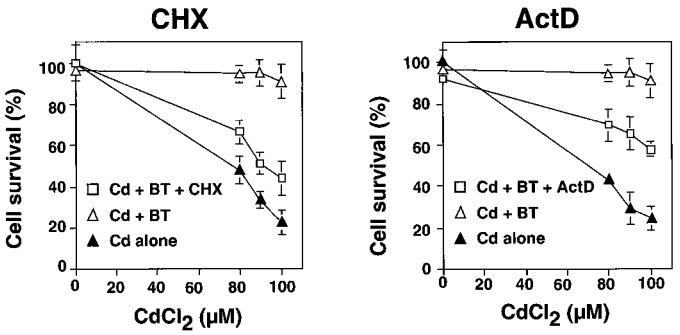

Fig. 6. Effects of cycloheximide and actinomycin D on the protective effect of betulin against the toxicity of cadmium in HepG2 cells. Cycloheximide (CHX; 2 μg/ml) and actinomycin D (ActD; 0.05 μg/ml) were added separately to cultures of HepG2 cells. Betulin (BT; 0.5 μg/ml) was added 6 h later. The medium was replaced by drug-free fresh medium 4 h after the addition of betulin. $CdCl_2$ (Cd) was added at indicated concentrations 20 h after replacement of the medium. Cell survival was determined at 24 h after the addition of $CdCl_2$. Each point represents the mean value of results from three cultures with S.D. values (bars).

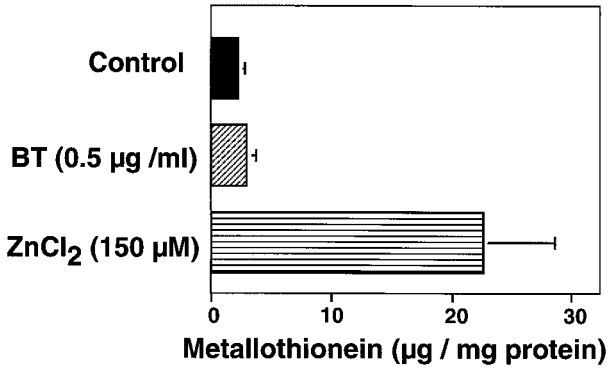

Fig. 7. Levels of MT in HepG2 cells after a 24-h incubation with betulin (BT) or $ZnCl_2$. Results are mean of results from four cultures with S.D. values (bars).

To investigate the relationship between the timing of treatment with betulin and the reduction in cadmium toxicity, we added betulin at 24, 6, and 0.5 h (regarded as simultaneous addition) before and 2 and 6 h after the addition of $CdCl_2$ and monitored its effects on cadmium toxicity. As shown in Fig. 3, when betulin and $CdCl_2$ were added simultaneously and when betulin was added after $CdCl_2$, there was almost no reduction in cadmium toxicity. The protective effect of betulin was detected only in pretreated cells. The effect tended to increase as the interval between the start of treatment with betulin and the addition of $CdCl_2$ was increased (Fig. 3). Because betulin significantly reduced cadmium toxicity only when it was added before $CdCl_2$, we next removed betulin from the medium after 2 h of cell culture in the presence of betulin and added $CdCl_2$ 22 h later; then, we measured cell survival. We found that a mere 2-h incubation in the presence of betulin (from 24 to 22 h before the addition of $CdCl_2$) markedly protected cells against cadmium toxicity (Fig. 4). These findings allow us to exclude the possibility that betulin might react with cadmium in the medium to inhibit cadmium toxicity. In fact, when molar equivalent amounts of betulin and $CdCl_2$ were mixed in the presence of culture medium in a test tube, no binding of $CdCl_2$ to betulin was detected, as determined by HPLC (data not shown). The possibility remains that betulin that accumulates in cells might bind cadmium only in the presence of some factor or factors within cells, thereby inhibiting cadmium toxicity. However, this possibility seems unlikely in view of the findings that the protective effect of betulin increased as the interval between the start of treatment with betulin and the addition of $CdCl_2$ became longer and that the toxicity was reduced by a mere 2-h incubation in the presence of betulin 24 h before the addition of $CdCl_2$. Our findings suggest that treatment with betulin has some effect on cell functions.

Because inhibition of the incorporation of cadmium into cells and/or promotion of the excretion of cadmium might have been caused by betulin, we investigated the effects of betulin on the accumulation of cadmium in cells. We found no difference between the amounts of cadmium accumulated by betulin-treated cells and that accumulated by DMSO-treated control cells (Fig. 5).

Next, to investigate the possibility that betulin might induce the synthesis of certain proteins induction in the cells,

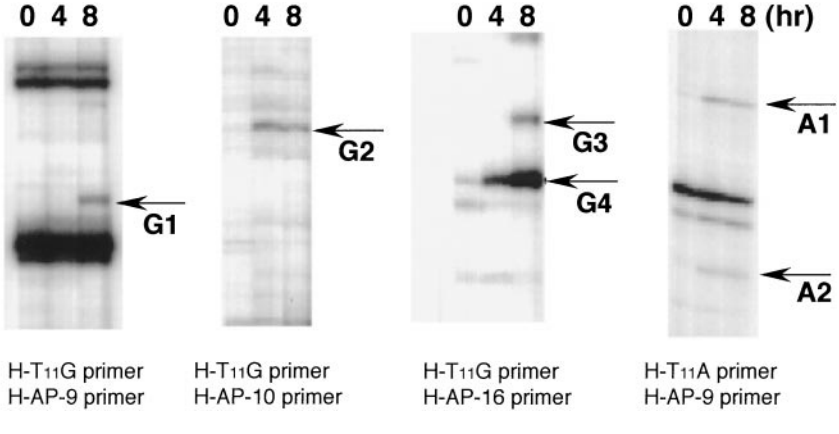

Fig. 8. Differential display of cDNAs derived from total cellular RNA of HepG2 cells that had been treated with betulin (4 μg/ml) for 0, 4, or 8 h. Bands of interest are indicated by arrows.

we investigated the effects of inhibitors of protein synthesis and RNA synthesis on the reduction of cadmium toxicity by betulin. When cells were treated with cycloheximide or with actinomycin D, the extent of the reduction of cadmium toxicity by betulin decreased markedly (Fig. 6). These findings suggest that betulin might promote the synthesis of a certain protein or proteins and that cadmium toxicity might be suppressed by such protein or proteins.

MT is an inducible protein that reduces heavy-metal toxicity (Bremner, 1987; Kägi, 1993). The synthesis of MT is induced by oleanolic acid (Liu et al., 1993b) and α-hederin (Liu et al., 1993a), which are known to reduce hepatotoxicity in mice. Therefore, we investigated the effects of betulin on the induction of MT synthesis. The concentration of MT in HepG2 cells was not significantly affected by betulin (Fig. 7). Zinc, which induces the synthesis of MT (Kägi, 1993), markedly increased the concentration of MT in cells (Fig. 7). However, the reduction of cadmium toxicity by $ZnCl_2$ was not as marked as that by betulin under the experimental conditions used in this study (data not shown). These findings suggest that betulin might reduce cadmium cytotoxicity by promoting the synthesis of some protein or proteins other than MT.

Finally, we used the differential display method (Liang and Pardee, 1992) to investigate whether betulin affects gene expression. Using 48 combinations of 3 anchored primers and 16 primers with arbitrary sequences, we applied the differential display method to our system and compared the pattern of gene expression in untreated cells with that in cells treated with betulin for 4 and 8 h. As shown in Fig. 8, the intensities of four bands (G2, G4, A1, and A2) were increased by treatment with betulin for both 4 and 8 h, and the intensities of two bands (G1 and G3) were increased by the 8-h treatment with betulin. Preliminary sequence analysis indicated that G1, one of these genes, is identical with human mitochondrial cytochrome *b* (Spurr and Bodmer, 1984; Marin-Garcia et al., 1995). Thus, betulin clearly enhanced the expression of several genes in human hepatocytes, and it appears that betulin reduces cadmium toxicity by promoting the synthesis of a certain protein or proteins that abolish the toxic effects of cadmium. The identification of a common betulin-responsive region in the promoter regions of the various genes might allow identification of the cadmium-resistance gene or genes. Furthermore, identification of the gene or genes involved in the reduction of cadmium toxicity by betulin might provide clues to the mechanism of cadmium toxicity, which remains unknown.

References

Bremner I (1987) Nutritional and physiological significance of metallothionein, in *Metallothionein II, Experientia Suppl* (Kagi JHR and Kojima Y eds) pp 81–107, Birkhauser Verlag, Basel.

Hikino H (1985) Antihepatotoxic activity of crude drugs. *Yakugaku Zasshi* **105:**109–118.

Kägi JHR (1993) Evolution, structure and chemical activity of class I metallothioneins: An overview, in *Metallothionein III* (Suzuki KT, Imura N and Kimura M eds) pp 29–55, Birkhäuser Verlag, Basel.

Liang P and Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science (Wash DC)* **257:**967–971.

Liu J, Choudhuri S, Liu Y, Kreppel H, Andrews GK and Klaassen CD (1993a) Induction of metallothionein by alpha-hederin. *Toxicol Appl Pharmacol* **121:**144–151.

Liu J, Liu Y and Klaassen CD (1994a) The effect of Chinese hepatoprotective medicines on experimental liver injury in mice. *J Ethnopharmacol* **42:**183–191.

Liu J, Liu Y, Mao Q and Klaassen CD (1994b) The effects of 10 triterpenoid compounds on experimental liver injury in mice. *Fundam Appl Toxicol* **22:**34–40.

Liu Y, Kreppel H, Liu J, Choudhuri S and Klaassen CD (1993b) Oleanolic acid protects against cadmium hepatotoxicity by inducing metallothionein. *J Pharmacol Exp Ther* **266:**400–406.

Ma XH, Zhao YC, Yin L, Han DW and Wang MS (1986) Studies on the preventive and therapeutic effects of ursolic acid on acute hepatic injury in rats. *Acta Pharmacol Sin* **21:**332–335.

Mahato SB, Sarkar SK and Poddar G (1988) Triterpenoid saponins. *Phytochemistry* **27:**3037–3067.

Marin-Garcia J, Ananthakrishnan R, Gonzalvo A and Goldenthal MJ (1995) Novel mutations in mitochondrial cytochrome b in fatal post partum cardiomyopathy. *J Inherit Metab Dis* **18:**77–78.

Miyairi S, Shibata S and Naganuma A (1998) Determination of metallothionein by high-performance liquid chromatography with fluorescence detection using an isocratic solvent system. *Anal Biochem* **258:**168–175.

Price KR, Johnson JT and Fenwick GR (1987) The chemistry and biological significance of saponins in foods and feedingstuffs. *CRC Crit Rev Food Sci Nutr* **26:**27–135.

Recio MC, Giner RM, Manez S, Gueho J, Julien HR, Hostettmann K and Rios JL (1995) Investigations on the steroidal anti-inflammatory activity of triterpenoids from *Diospyros leucomelas*. *Planta Med* **61:**9–12.

Shaikh ZA, Vu TT and Zaman K (1999) Oxidative stress as a mechanism of chronic cadmium-induced hepatotoxicity and renal toxicity and protection by antioxidants. *Toxicol Appl Pharmacol* **154:**256–263.

Spurr NK and Bodmer WF (1984) Serendipitous cloning of a mitochondrial cDNA and its polymorphism. *Mol Biol Med* **2:**239–249.

Steele JC, Warhurst DC, Kirby GC and Simmonds MS (1999) In vitro and in vivo evaluation of betulinic acid as an antimalarial. *Phytother Res* **13:**115–119.

Sun IC, Shen JK, Wang HK, Cosentino LM and Lee KH (1998a) Anti-AIDS agents. 32. Synthesis and anti-HIV activity of betulin derivatives. *Bioorg Med Chem Lett* **8:**1267–1272.

Sun IC, Wang HK, Kashiwada Y, Shen JK, Cosentino LM, Chen CH, Yang LM and Lee KH (1998b) Anti-AIDS agents. 34. Synthesis and structure-activity relationships of betulin derivatives as anti-HIV agents. *J Med Chem* **41:**4648–4657.

Tokuda H, Ohigashi H, Koshimizu K and Ito Y (1986) Inhibitory effects of ursolic acid and oleanoic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Lett* **33:**279–285.

Webb M (1979) *The Chemistry, Biochemistry and Biology of Cadmium*, Elsevier/North Holland, Amsterdam.

Send reprint requests to: Prof. Akira Naganuma, Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan. E-mail: naganuma@mail.pharm.tohoku.ac.jp