Biochemical Pharmacology, Vol. 43, No. 10, pp. 2111–2115, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

LIPID PEROXIDATION AND IRREVERSIBLE DAMAGE IN THE RAT HEPATOCYTE MODEL

PROTECTION BY THE SILYBIN–PHOSPHOLIPID COMPLEX IdB 1016

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(*Received* 13 *September* 1991; *accepted* 20 *February* 1992)

Abstract—IdB 1016 is a new silybin–phospholipid complex which is more bioavailable than the flavonoid silybin itself and displays free radical scavenging and antioxidant properties in liver microsomes. We report here that the addition of increasing concentrations of IdB 1016 to isolated rat hepatocytes caused a dose-dependent inhibition of lipid peroxidation induced by ADP-Fe^{3+} or cumene hydroperoxide. Moreover, IdB 1016 at the concentration which completely prevented MDA formation also protected isolated hepatocytes against the toxicity of pro-oxidant agents such as allyl alcohol, cumene hydroperoxide and bromotrichloromethane, without interfering with the activation mechanism of these xenobiotics. Similar protection was also obtained in hepatocytes prepared from animals pretreated *in vivo* with IdB 1016 while rat supplementation with pure silybin was totally inefficient. These results indicate IdB 1016 as being a potentially useful protective agent against free radical-mediated toxic liver injury.

Silybin is the main active compound present in silymarin, a purified extract from *Silybum marianum*, which has been used for over 20 years in clinical practice for the treatment of toxic liver diseases [1, 2].

In vitro addition of silybin to isolated hepatocytes or *in vivo* pretreatment of experimental animals with this flavanolignan exerts antioxidant effects and prevents liver damage induced by several hepatotoxic agents [3, 4]. Interestingly, the effects of silybin are greater after parenteral than oral administration probably because of the poor bioavailability of orally given silybin [5]. Complexing this flavanolignan with phosphatidylcholine in a compound known as IdB 1016 has been shown to greatly improve enteral absorption of silybin [5, 6].

We have reported previously that a single oral administration of IdB 1016 to rats is able to prevent lipid peroxidation induced in liver microsomal preparations by a subsequent *in vitro* addition of pro-oxidant compounds [7]. This effect is related to the capacity of the drug to scavenge lipodienyl radicals as well as other carbon centered free radicals (methyl and trichloromethyl radicals) as indicated by experiments using ESR spectroscopy associated with the spin trapping technique [7].

In order to evaluate the possible relationship between the antioxidant properties of IdB 1016 and its protective action against liver injury, we have investigated the effects of the *in vitro* and *in vivo* administration of the drug on lipid peroxidation and cell death induced in isolated hepatocytes by three pro-oxidant compounds, namely bromotrichloromethane ($CBrCl_3$†), allyl alcohol (AA) and cumene hydroperoxide (CuOOH).

MATERIALS AND METHODS

Male Sprague–Dawley rats, 200–250 g body wt, were obtained from Nossan (Correzzana, Italy) and fed on a semi-synthetic diet containing 40 IU of α-tocopherol and 12,000 IU of vitamin A/kg, with free access to water. IdB 1016 and pure silybin were suspended in water and administered intragastrically to rats at a dose of 1.5 and 0.6 g/kg body wt, respectively, 1 hr before killing.

Collagenase Type I, ethylene-glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 4-(2-hydroxyethyl)-1 piperazine-ethane sulfonic acid (HEPES) and adenosine-5′-diphosphate were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.); CuOOH, AA and $CBrCl_3$ were from Fluka AG (Buchs, Switzerland); and β-glucuronidase/arylsulfatase was from Boehringer Biochemia (Mannheim, F.R.G.). Extralut 3 solid extraction columns and the LiChrosorb-diol HPLC column were purchased from Merck (Darmstadt, Germany). All other chemicals were from Merck (Darmstadt, Germany) and BDH Chemicals (Poole, U.K.).

Hepatocytes were isolated from both untreated and IdB 1016 or silybin-pretreated rats by the collagenase perfusion technique as described in [8]. Cells were then suspended in a HEPES-buffered salt solution, pH 7.4 [8], at a concentration of 5×10^6 cells/mL. Aliquots of 20 mL of hepatocyte suspension were preincubated for 30 min at 37° in the presence

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† Abbreviations: $CBrCl_3$, bromotrichloromethane; AA, allyl alcohol; CuOOH, cumene hydroperoxide; HEPES, 4-(2-hydroxyethyl)-1 piperazine-ethane sulfonic acid; LDH, lactate dehydrogenase; MDA, malonaldehyde; GSH, glutathione.

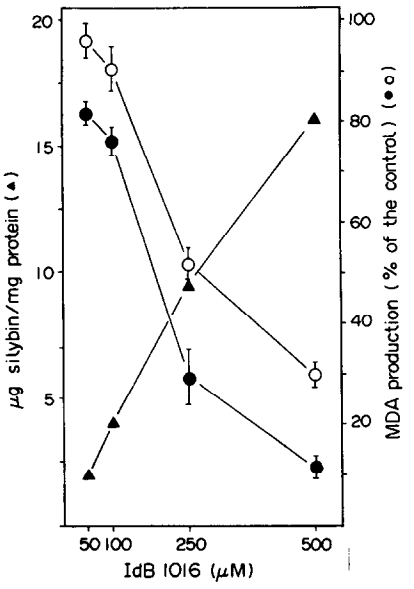

Fig. 1. Effect of hepatocyte pretreatment with increasing concentrations of IdB 1016 (50, 100, 250 and 500 μM IdB 1016 corresponding to 19.5, 39, 97.5 and 195 μM silybin, respectively) on the intracellular concentration of silybin and on lipid peroxidation. Isolated hepatocytes were preincubated for 30 min with different concentrations of IdB 1016 and then incubated for 60 min with 2.5 mM ADP/0.1 mM $FeCl_3$ or 0.5 mM CuOOH. Amount of silybin recovered in the hepatocytes (▲); formation of MDA following addition of ADP/$FeCl_3$ (○) or CuOOH (●). MDA production is expressed as percentage over the amounts recovered in cells not pretreated with the flavonoid and corresponding to 3.36 ± 0.07 and 2.8 ± 0.3 nmol/10^6 cells for ADP/$FeCl_3$ and CuOOH-treated cells, respectively. MDA values are means of three experiments ± SEM. Intracellular silybin values are means of two experiments in triplicate.

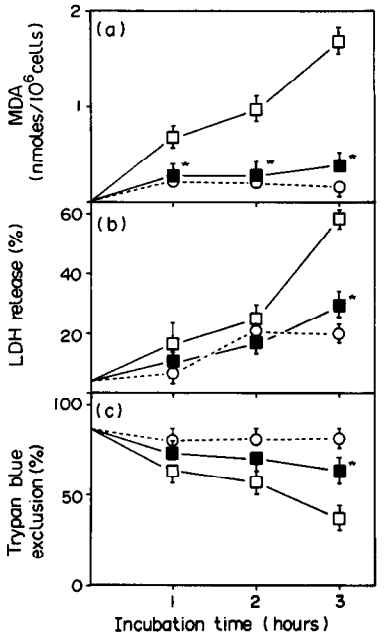

Fig. 2. Effect of 30 min cell pretreatment with 0.5 mM IdB 1016 (i.e. 195 μM silybin) on MDA production (a), LDH release (b) and Trypan blue exclusion (c) for up to 3 hr incubation with 0.25 μL of $CBrCl_3$ under the conditions described in Materials and Methods. Values are means of 5–6 experiments ± SEM. Values from non-intoxicated cell suspension ± IdB 1016 have been pooled in a single control group. Control (○), $CBrCl_3$ (□), $CBrCl_3$ + IdB 1016 (■). Statistically different as to $CBrCl_3$ vs $CBrCl_3$ + IdB 1016 ($P < 0.05$).

of different concentrations of IdB 1016 dissolved in dimethyl sulfoxide (5 μL/mL of cell suspension). The same amount of dimethyl sulfoxide when added to controls did not change any of the tested parameters. $CBrCl_3$ (0.25 μL) was added to the central well of stoppered 50-mL flasks containing 1.5 mL of cell suspension and allowed to diffuse in the closed system. ADP/$FeCl_3$ (2.5–0.1 mM), CuOOH (0.5 mM) or AA (0.8 mM) were added directly to 4 mL of cell suspension.

Cell viability was assessed both by the Trypan blue exclusion test and by measuring the release of lactate dehydrogenase (LDH) as reported previously [8].

Lipid peroxidation was measured as malonaldehyde (MDA) accumulation in cell suspension aliquots using the thiobarbituric acid reaction according to Bernheim *et al.* [9]. Under these conditions the chromogen formation is essentially due to the MDA and not to other reacting materials, as verified by HPLC analysis [10].

Total free thiols were measured by the colorimetric reaction with 5,5′-dithio-bis-2-nitrobenzoic acid according to Sedlack and Lindsay [11].

The intracellular concentration of silybin was measured in aliquots of hepatocyte suspension (4 mL, i.e. 20 × 10^6 cells) incubated for 30 min with different amounts of IdB 1016. After incubation, hepatocytes were harvested by 1 min centrifugation at 1000 rpm and resuspended with fresh HEPES-buffered salt solution. Cells were washed with this solution twice in order to remove extracellular silybin. The packed cells corresponding to a volume of about 0.1 mL were mixed with 0.4 mL of 0.25 M sucrose buffered with 10 mM HEPES and then lysed by repeated freezing and thawing. After lysis, 0.1 mL of the final suspension was added to 1.2 mL of 0.2 M acetate buffer (pH 5), 40 μL of 1 N HCl and the mixture incubated for 48 hr at 37° in the presence of 80 μL β-glucuronidase/arylsulfatase (5.5/2.5 U/mL) in order to convert all esterified silybin into the free form. Samples were then diluted with 0.1 M citrate buffer (pH 4), placed on Extrelut 3 solid extraction columns and eluted with *t*-butylmethylether.

Silybin identification was achieved by HPLC analysis at 214 nm using a LiChrosorb-Diol 150 × 3 normal phase-column and a mobile-phase consisting of 70% *n*-hexane and 30% ethanol acidified with 85% phosphoric acid (0.12 mL/L) as reported previously [12]. Quantification was obtained by processing under identical conditions authentic silybin standards. Results were expressed in terms

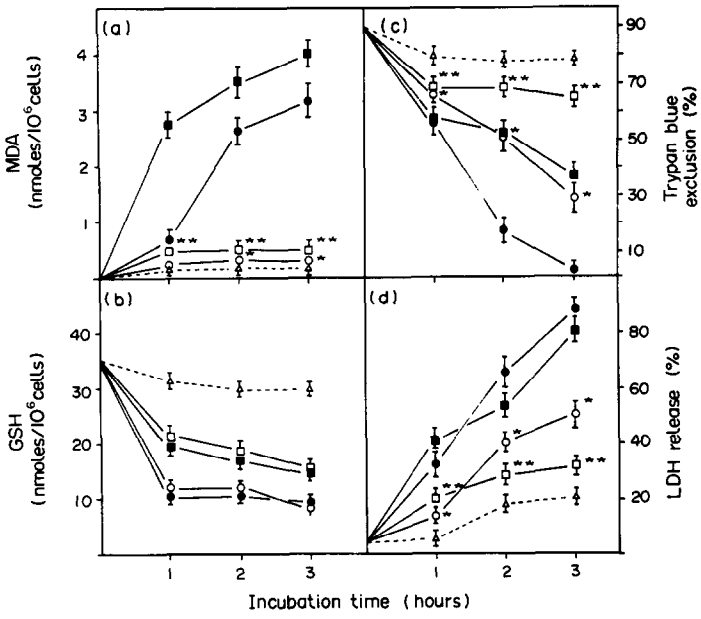

Fig. 3. Effect of 30 min cell pretreatment with 0.5 mM IdB 1016 (i.e. 195 μM silybin) on MDA production (a), GSH intracellular content (b), Trypan blue exclusion (c) and LDH release (d) for up to 3 hr incubation with 0.8 mM AA or 0.5 mM CuOOH. Values are means of 5–6 experiments ± SEM. Control (△), AA (●), AA + IdB 1016 (○), CuOOH (■), CuOOH + IdB 1016 (□). * Statistically different as to AA vs AA + IdB 1016 and ** statistically different as to CuOOH vs CuOOH + IdB 1016 ($P \leq 0.05$).

of free silybin/mg protein. Cellular protein content was determined by the method of Lowry *et al.* [13].

Statistical evaluations were performed by using the Student's *t*-test.

RESULTS AND DISCUSSION

Preincubation of isolated hepatocytes with increasing amounts of IdB 1016 caused a linear rise in the intracellular concentration of silybin (Fig. 1) which was associated with a dose-dependent decrease in lipid peroxidation as induced by incubating hepatocytes with either 2.5 mM ADP and 0.1 mM $FeCl_3$ or 0.5 mM CuOOH. The amount of silybin added to the hepatocytes with 0.5 mM IdB 1016 (i.e. 195 μM silybin) was very close to that detected in the plasma of rats (229 μM silybin) 1 hr after rat oral treatment with IdB 1016 at 1.5 g/kg body wt [7]. Therefore, this dose was used to evaluate the effect of IdB 1016 on the cellular damage induced by three pro-oxidant substances, i.e. AA, CuOOH and $CBrCl_3$.

$CBrCl_3$ toxicity depends mainly upon the formation of free radical species which are able to alkylate cell macromolecules and initiate lipid peroxidation [14]. Cell damage induced by AA is known to depend on its metabolism by cytosolic alcohol dehydrogenase producing acrolein which reacts actively with sulphydryl groups [15, 16], leading to a marked glutathione (GSH) depletion and a subsequent stimulation of lipid peroxidation [15, 16]. Cell damage caused by organic hydroperoxides follows their intracellular degradation which triggers the oxidation of glutathione, protein thiols and pyridine nucleotides [17, 18].

As shown in Figs 2a and 3a, 30 min preincubation of isolated hepatocytes with 0.5 mM IdB 1016 (i.e. 195 μM silybin) completely prevented MDA production induced by $CBrCL_3$, AA and CuOOH. Conversely, IdB 1016 did not protect against the depletion of hepatocyte GSH induced by AA and CuOOH, indicating that IdB 1016 did not interfere with the specific events responsible for the onset of oxidative injury (Fig. 3b). No change in GSH intracellular concentration was observed when hepatocytes were intoxicated with $CBrCl_3$ (data not shown).

The irreversible cell injury caused by the three toxins was evaluated in terms of release of LDH and Trypan blue exclusion. In the case of $CBrCl_3$ and CuOOH poisoning, cell preincubation with IdB 1016 almost completely abolished LDH leakage and Trypan blue uptake for up to 3 hr treatment (Figs 2c, d and 3c, d). Conversely, the supplementation with IdB 1016 decreased by only about 50% cell injury induced by AA (Fig. 3c, d).

The above experiments indicated that the antihepatotoxic action of IdB 1016 added *in vitro* to the hepatocyte suspension was related to the antioxidant effect. Thus, we decided to evaluate whether a comparable effect was also reproducible in hepatocytes obtained from rats orally supplemented with 1.5 g/kg body wt IdB 1016, i.e. with a dose of antioxidant demonstrated to decrease strongly lipid peroxidation in liver microsomes [7].

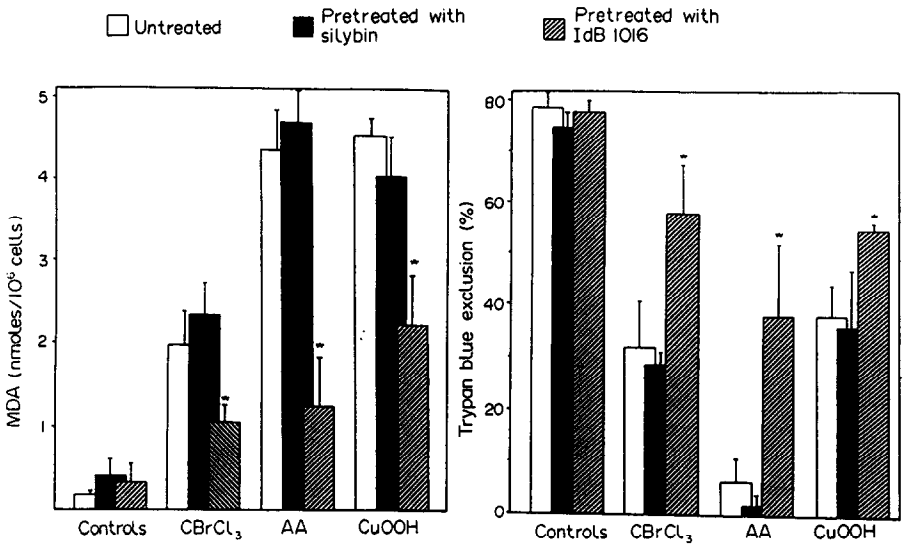

Fig. 4. Effect of *in vivo* oral administration of IdB 1016 or pure silybin on the hepatotoxic effect of $CBrCl_3$, AA or CuOOH. Hepatocytes were isolated 1 hr after the intragastric administration of IdB 1016 or pure silybin at the doses of 1.5 g/kg body wt and 0.6 g/kg body wt, respectively. MDA production (left panel) and Trypan blue exclusion (right panel) were evaluated after 3 hr incubation of isolated hepatocytes with $CBrCl_3$ (0.25 μL as reported in Materials and Methods), AA (0.8 mM) or CuOOH (0.5 mM). Values are means of five different experiments ± SEM. * Statistically different as to untreated and silybin-pretreated hepatocytes vs IdB 1016-pretreated hepatocytes ($P < 0.05$).

Figure 4 shows that the *in vivo* administration of IdB 1016 at least halved, in the subsequently isolated hepatocytes, the MDA production stimulated by 3 hr incubation with either $CBrCl_3$, AA or CuOOH. Furthermore, the same pretreatment lowered by 50, 80 and 31% the loss of cell viability caused by $CBrCl_3$, AA and CuOOH, respectively (Fig. 4). It is noteworthy that no protection against MDA production and Trypan blue staining was observed when an equivalent amount of uncomplexed silybin was administered orally to rats instead of IdB 1016 (Fig. 4). This indicates an increased bioavailability of silybin when it is complexed with phospholipids.

As reported recently by Letteron *et al.* [19], the parenteral administration to mice of silymarin, but not of silybin dihemisuccinate, decreases hepatic lipid peroxidation and protects against liver damage following carbon tetrachloride poisoning *in vivo*. One possible explanation for such a discrepancy is that silybin was administered in the form of a pro-drug i.e. dihemisuccinate [20]. In any case, silybin does not appear sufficiently bioavailable *per se* as also demonstrated by the present work. Another possibility is that silymarin contains some other active compounds responsible for the protection against CCl_4 toxicity. In the same study it was reported that both silymarin and silybin interfered with different hepatic monooxygenase activities [19]. This is not the case with IdB 1016 since we have shown previously that IdB 1016 administration does not change cytochrome P450-mediated aminopyrine demthylase and ethoxycoumarine deethylase activities in liver microsomes [7]. Moreover, the amounts of silybin administered here as IdB 1016 are much lower than those reported by Letteron *et al.* [19].

We have reported in a recent work [7] that IdB 1016 displays an efficient scavenging activity not only against the trichloromethyl radical ($CCl_3\cdot$) involved in $CBrCl_3$ toxicity [14, 21] but also against lipodienyl radicals involved in all lipid peroxidation processes. This latter effect might be relevant in understanding the protection exerted by IdB 1016 against the three toxins investigated here.

Although the mechanism by which lipid peroxidation can contribute to cell injury due to pro-oxidant compounds has not yet been completely clarified [14, 22], studies in our laboratory have shown that lipid peroxidation favours the entrance of Ca^{2+} into hepatocytes [23]. Consistently, the inhibition of lipid peroxidation by vitamin E supplementation prevents Ca^{2+} accumulation in the liver [24] and reduces the extent of inactivation of plasma membrane Ca^{2+}, Mg^{2+} ATPase [25]. Moreover, the antioxidant *N,N'*-diphenyl-*p*-phenylenediamine has been reported to protect against oxidative cell damage induced by *t*-butylhydroperoxide by preventing the loss of mitochondrial membrane potential [26]. Thus, through its antioxidant action, IdB 1016 might prevent the onset of the above-mentioned alterations which are thought to play an important role in the pathogenesis of toxic liver damage [22].

In conclusion, the present results confirm in the isolated hepatocyte model the bioavailability and antioxidant activity of IdB 1016, observed previously using rat liver microsomes [7]. Moreover, it is

demonstrated here for the first time that IdB 1016 can counteract the hepatotoxic effect of several different compounds acting through oxidative mechanisms, indicating the potential usefulness of the compound as hepatoprotective agent.

Acknowledgements—This work has been supported by Centro Nazionale delle Ricerche, Targeted Project "Prevention and Control Disease Factors", Subproject "Qualità dell'Ambiente e Salute" Contract No. 91.00142.PF41 and by the Ministry of the University, Roma, Italy.

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