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INHIBITION OF RAT LIVER MICROSOMAL LIPID PEROXIDATION BY BOLDINE

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Abstract—The alkaloid boldine, found in the leaves and bark of boldo, was an effective inhibitor of rat liver microsomal lipid peroxidation under a variety of conditions. The following systems all displayed a similar sensitivity to boldine: non-enzymatic peroxidation initiated by ferrous ammonium sulfate; iron-dependent peroxidation produced by ferric–ATP with either NADPH or NADH as cofactor; organic hydroperoxide-catalyzed peroxidation; and carbon tetrachloride plus NADPH-dependent peroxidation. Boldine inhibited the excess oxygen uptake associated with microsomal lipid peroxidation. Thus, boldine was effective in inhibiting iron-dependent and iron-independent microsomal lipid peroxidation, with 50% inhibition occurring at a concentration of about 0.015 mM. Boldine did not appear to react efficiently with superoxide radical or hydrogen peroxide, but was effective in competing for hydroxyl radicals with chemical scavengers. Concentrations of boldine which produced nearly total inhibition of lipid peroxidation had no effect on microsomal mixed-function oxidase activity nor did boldine appear to direct electrons from NADPH–cytochrome P450 reductase away from cytochrome P450. Boldine completely protected microsomal mixed-function oxidase activity against inactivation produced by lipid peroxidation. The effectiveness of boldine as an anti-oxidant under various conditions, and its low toxicity, suggest that this alkaloid may be an attractive agent for further evaluation as a clinically useful anti-oxidant.

In view of the increasing interest in reactive oxygen species and free radical reactions, numerous compounds, natural and synthetic, have been studied for their anti-oxidant activities. Boldine§ ([*S*]-2,9-dihydroxy-1,10-dimethoxyaporphine) is the major alkaloid found in the leaves and bark of boldo (*Peumus boldus Mol*) [1], a common evergreen tree found in Chile. The ready abundance of boldo, the high concentration of boldine in boldo bark, the very low toxicity [2] and the lack of genotoxic and mutagenic activities in prokaryotic and eukaryotic cells [3] make this alkaloid a readily available, inexpensive compound, which possesses anti-oxidant activity [4, 5]. Recent results of Speisky *et al.* [4, 5] have demonstrated that boldine displays high reactivity towards free radicals. For example, the auto-oxidation of rat brain homogenates as detected by luminescence, oxygen uptake and production of thiobarbituric acid (TBA)-reactive components is inhibited by boldine with an apparent K_I of about 19–30 μM [4]. Peroxidation of red blood cell plasma membrane by alkylperoxyl radicals generated from the thermolysis of 2,2′-azobis-2-amidinopropane [6] is decreased by boldine, with an apparent K_I of about 13 μM [4]. Such anti-oxidant efficiency is comparable to that of the widely used bioflavonoid (+)-cyanidanol-3 and exceeds that of silybin [7]. The spontaneous and the ferrous-induced peroxidation of fish oil is efficiently prevented by boldine [5]; the effectiveness of boldine exceeds that of α-tocopherol, butylated hydroxytoluene and butylated hydroxyanisole. These results suggest that boldine can react with alkylperoxyl radicals, lipid radicals, or both, and the anti-oxidant properties of boldine may play a role in its therapeutic use. Lanhers *et al.* [8] showed that boldine, in a concentration range of about 0.02 to 0.08 mM, exerts protection against *t*-butyl hydroperoxide (t-BHP)-induced damage to isolated liver cells and against carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice *in vivo*.

The current study was carried out to assess the effectiveness of boldine in preventing lipid peroxidation of microsomes by a variety of systems, e.g. iron-dependent and iron-independent, enzymatic or non-enzymatic, and systems requiring metabolism of the xenobiotic inducer by cytochrome P450. The effect of boldine on microsomal mixed-function oxidase activity was determined, as well as the ability of boldine to interact with reactive oxygen intermediates such as superoxide anion radical and H_2O_2, in order to gain insight into the specificity of the alkaloid as an anti-oxidant.

MATERIALS AND METHODS

Liver microsomes were isolated from male Sprague–Dawley rats (weighing about 150 g) that were treated with pyrazole to induce cytochrome P4502E1 [9], which is very reactive with CCl_4 [10]

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§ Abbreviations: boldine, [*S*]-2,9-dihydroxy-1,10-dimethoxyaporphine; H_2O_2, hydrogen peroxide; CCl_4, carbon tetrachloride; DMN, dimethylnitrosamine; TBA, thiobarbituric acid; KMB, 2-keto-4-thiomethylbutyric acid; ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; and t-BHP, *tert*-butyl hydroperoxide.

and in oxidizing substrates such as ethanol or dimethylnitrosamine (DMN) [11, 12]. Microsomes were prepared by differential centrifugation, washed twice, and stored in 125 mM KCl at −70°. Boldine was extracted and crystallized as previously described [4, 5]. Stock solutions were prepared fresh and protected from light. The boldine was dissolved in HCl, pH 1–2, as a 10 mM stock solution and then slowly neutralized with stirring to pH 5–7 with NaOH.

Iron-dependent microsomal lipid peroxidation was determined in a reaction system consisting of 50 mM Tris, pH 7.4, 25 μM ferric–ATP (1:20 chelate) and about 0.5 mg microsomal protein in a final volume of 1 mL. Reactions were initiated by the addition of either NADPH or NADH (0.8 mM final concentration) at 37° and were terminated after 2.5 to 10 min (NADPH) or 10 to 30 min (NADH) by the addition of trichloroacetic acid (4.5% final concentration). The production of TBA-reactive material [13] was taken as a reflection of lipid peroxidation. In some experiments, the NADH or NADPH cofactor was omitted and ferrous-dependent lipid peroxidation was initiated by the addition of ferrous ammonium sulfate (1 mM final concentration) prepared fresh in argon-sparged water. Iron-independent microsomal lipid peroxidation was determined in two systems: t-BHP (final concentration of 3 mM, reaction time of 5 min) or CCl_4 (2 mM final concentration) plus NADPH. Microsomal oxygen consumption was determined with a Clark oxygen electrode and a Yellow Springs oxygen monitor.

Oxidation of ethanol to acetaldehyde was determined in a reaction system containing 100 mM potassium phosphate buffer, pH 7.4, 0.5 mM sodium azide (to inhibit contaminating catalase), about 0.5 mg microsomal protein and 100 mM ethanol in a final volume of 0.5 mL. Reactions were initiated by the addition of NADPH (0.8 mM final concentration) and were carried out at 37° for 7 min before termination by addition of HCl to a final concentration of 0.5 N. Acetaldehyde was determined by a headspace, gas chromatography procedure [14]. Oxidation of DMN to formaldehyde was determined in a similar reaction system except for omission of the azide, and replacement of ethanol by DMN (2 mM final concentration). Reactions were carried out for 20 min at 37°, and terminated by the addition of trichloroacetic acid [4.5% (w/v) final concentration]; formaldehyde was assayed by the method of Nash [15]. Boldine had no effect on the recovery of standard amounts of either acetaldehyde or formaldehyde. The NADPH-dependent generation of H_2O_2 by microsomes was determined by assaying the oxidation of methanol to formaldehyde by the catalase–H_2O_2 compound I complex [16]. Reactions were carried out as previously described [17], in the absence and presence of 25 μM ferric–EDTA (1:2 chelate).

The reduction of cytochrome *c* by superoxide anion radical was studied in a reaction system consisting of 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 0.5 mM hypoxanthine, and 0.05 mM ferricytochrome *c* in a final volume of 1 mL. Reactions were initiated by the addition of 0.01 U of xanthine oxidase, and the increase in absorbance at 550 nm was determined. The oxidation of ethanol to acetaldehyde by catalase–H_2O_2 was determined in a reaction system containing 100 mM potassium phosphate, pH 7.4, 100 mM ethanol, 0.01 U xanthine oxidase and 6500 U catalase in a final volume of 1 mL. Reactions were initiated by the addition of hypoxanthine (0.5 mM final concentration) and terminated after 20 min with HCl (final concentration of 0.5 N). The amount of acetaldehyde produced was determined by headspace gas chromatography [14]. The production of ethylene gas from 2-keto-4-thiomethylbutyric acid (KMB) was assayed as an index of generation of ·OH-like species [14]. The oxidation of 1 mM KMB to ethylene was determined in the xanthine oxidase system described above except for the omission of catalase, but with the addition of 50 μM ferric–EDTA (1:2 chelate) as the iron catalyst for the Haber–Weiss reaction [18].

Experiments were carried out in duplicate or triplicate and with at least two different microsomal preparations. All values have been corrected for zero-time controls in which acid was added prior to initiation of the reaction with cofactor. The buffers and water used to prepare solutions were passed through columns of Chelex-100 resin to remove metal contaminants. All reagents were of the highest grade available.

RESULTS

Studies with xanthine oxidase. Initial experiments evaluated the effectiveness of boldine in reacting with superoxide radical, produced during the oxidation of hypoxanthine by xanthine oxidase. Reduction of cytochrome *c* by this system was strongly inhibited by superoxide dismutase (SOD), indicating that the reduction was largely mediated by superoxide (Table 1). Boldine itself neither reduced cytochrome *c* nor had any effect on xanthine oxidase activity as determined by the increase in absorbance at 293 nm. Boldine, at concentrations up to 1 mM, had no effect on the reduction of cytochrome *c* by xanthine oxidase (Table 1), suggesting that even at concentrations 20-fold greater than the detector, there was little competition for, or interaction with, superoxide.

The ability of boldine to inhibit the production of acetaldehyde when ethanol is oxidized by catalase–H_2O_2 was studied to determine if boldine interacts with H_2O_2 or inhibits the peroxidatic activity of catalase. The H_2O_2 was generated from the oxidation of hypoxanthine by xanthine oxidase. Boldine was a weak inhibitor of acetaldehyde oxidation by this system (Table 1) as only 30% inhibition was observed at a boldine concentration as high as 1 mM. By contrast, 1 mM azide, a known inhibitor of peroxidatic activity of catalase, produced 90% inhibition. These results suggest that boldine does not readily interact directly with H_2O_2.

Oxidation of hypoxanthine by xanthine oxidase in the presence of ferric–EDTA produces ·OH-like species which can readily be detected by the oxidation of chemical scavengers such as KMB. Results in Table 2 show that boldine produced a concentration-

Table 1. Evaluation of the interaction of boldine with superoxide, and H_2O_2

Addition	Concentration (mM or U)	Cytochrome *c* reduction (nmol/min)	Acetaldehyde production Catalase-H_2O_2 (nmol/min)
Control		14.3	22
Boldine	0.1	15.3	19
	0.2	15.6	18.5
	0.5	14.7	16.5
	1.0	14.8	15.6
SOD	43 U	4.6	
SOD	86 U	2.2	
Azide	1.0		2.3

Superoxide production was determined by assaying the reduction of cytochrome *c* by a xanthine oxidase system. Oxidation of ethanol to acetaldehyde by catalase was determined in the presence of hypoxanthine plus xanthine oxidase to generate the H_2O_2.

Table 2. Effect of boldine on hydroxyl radical-dependent oxidation of KMB to ethylene

Addition	Concentration (mM)	Ethylene production (nmol/min)	Effect of addition (%)
Control		1.37	
Boldine	0.1	1.24	−9
	0.5	0.72	−47
	1.0	0.56	−59
	2.0	0.38	−72
	5.0	0.21	−85
	8.0	0.11	−92
Ethanol	1.0	1.41	−3
	2.0	1.34	−2
	5.0	1.04	−24
	8.0	0.87	−36
DMSO	1.0	0.79	−42
	2.0	0.47	−66
	5.0	0.33	−76
	8.0	0.26	−81

The oxidation of 1 mM KMB to ethylene by the xanthine oxidase reaction was determined as described in Materials and Methods in the presence of 0.05 mM ferric–0.10 mM EDTA as the iron catalyst, and the indicated additions.

dependent inhibition of the oxidation of 1 mM KMB to ethylene. At an equimolar concentration of boldine and KMB, ethylene production was inhibited by 59%. For comparative purposes, the effects of two known ·OH scavengers, ethanol and dimethyl sulfoxide (DMSO), were evaluated. Ethanol, with a rate constant for interacting with ·OH of $1.1 \times 10^9\,M^{-1}\,sec^{-1}$ [19], was a weak inhibitor of KMB oxidation as only 36% inhibition was observed at an ethanol to KMB ratio of 8:1 (Table 2). DMSO, with a rate constant of $7 \times 10^9\,M^{-1}\,sec^{-1}$ [19], was a much better inhibitor than ethanol (Table 2). Boldine was considerably more effective than ethanol in competing with KMB for ·OH; in fact, boldine was slightly more effective than DMSO, suggesting that the alkaloid is a powerful ·OH scavenger, with a rate constant for interacting with ·OH that is close to diffusion control.

Microsomal studies. To evaluate whether boldine can interact with major components of the mixed-function oxidase system such as NADPH–cytochrome P450 reductase and cytochrome P450, the effect of the alkaloid on oxidation of two substrates, ethanol and DMN, which are actively oxidized by cytochrome P4502E1 [11, 12], was determined. At concentrations up to 0.1 mM, boldine had no effect on the oxidation of the two substrates (Table 3). Boldine had no effect on reductase activity as determined by NADPH–cytochrome *c* reductase activity, and it neither interfered with the reduced P450-carbon monoxide binding spectrum nor produced any P420 (data not shown). Microsomal production of H_2O_2 largely reflects the decay of oxygenated cytochrome P450 intermediates produced during the mixed-function oxidation cycle [20], whereas ferric–EDTA increases H_2O_2 production by interaction with the reductase [21]. Boldine had no effect on microsomal production of H_2O_2 in the absence or the presence of ferric–EDTA (Table 3).

Table 3. Effect of boldine on rat liver microsomal oxidation of ethanol and DMN and on microsomal generation of H_2O_2

Concentration of boldine	Oxidation of ethanol	Oxidation of DMN	H_2O_2 production	
			−FeEDTA	+FeEDTA
(μM)	(nmol/min/mg microsomal protein)			
0	16.6 ± 1.3	6.51 ± 1.2	3.19	9.01
10	15.8 ± 2.2	6.06 ± 1.1		
20			3.50	8.14
30	16.8 ± 1.7	6.29 ± 1.3		
50	16.4 ± 1.9	5.82 ± 0.2		
100	16.8 ± 2.7	5.48 ± 0.7	3.21	8.81

NADPH-dependent microsomal oxidation of ethanol to acetaldehyde or DMN to formaldehyde was assayed as described in Materials and Methods in the presence of the indicated concentrations of boldine. Microsomal production of H_2O_2 was determined in the absence (largely cytochrome P450 mediated) and presence of 25 μM ferric–EDTA (largely NADPH–cytochrome P450 reductase) by determining the production of formaldehyde when methanol was oxidized by catalase plus H_2O_2. Results are given either as the mean from two experiments (H_2O_2 production) or as the mean ± SEM from three experiments (oxidation of ethanol or of DMN).

Table 4. Effect of boldine on iron-dependent microsomal lipid peroxidation

Concentration of boldine (mM)	Microsomal lipid peroxidation (nmol/min/mg protein)		
	NADPH + Ferric	NADH + Ferric	Ferrous
0	1.07	0.46	0.46
0.005	0.85 (21)	0.38 (17)	0.30 (35)
0.010	0.62 (42)	0.29 (37)	0.18 (61)
0.015	0.47 (56)	0.23 (50)	0.10 (78)
0.020	0.38 (64)	0.17 (63)	0.09 (80)
0.030	0.18 (83)	0.09 (80)	0.02 (96)

Microsomal production of TBA-reactive material was determined in the following three systems; NADPH plus ferric–ATP, NADH plus ferric–ATP, and ferrous ammonium sulfate, in the absence and presence of the indicated concentrations of boldine. Numbers in parentheses refer to the percent inhibition by boldine.

Microsomal lipid peroxidation. Ferric–ATP is an effective iron complex which catalyzes microsomal lipid peroxidation in the presence of reductants such as NADPH or NADH [17, 22, 23]. Rates of production of TBA-reactive material were about 2-fold greater with NADPH than NADH (Table 4). Boldine was an effective inhibitor of NADPH- and NADH-dependent production of TBA-reactive material (Table 4). Under these conditions, microsomal lipid peroxidation was inhibited 50% at boldine concentrations of about 0.015 mM. Although the above studies indicated that boldine does not inhibit enzymatic components of the mixed-function oxidase system which are necessary for lipid peroxidation to occur with ferric-chelate plus NADPH, non-enzymatic lipid peroxidation using ferrous ammonium sulfate in the absence of cofactor was also studied. Boldine proved to be a very effective inhibitor of ferrous-catalyzed non-enzymatic lipid peroxidation (Table 4).

The above lipid peroxidation studies involved iron as the metal ion catalyst. It was possible that boldine inhibited lipid peroxidation by complexing iron in a form which did not catalyze lipid peroxidation. Therefore, iron-independent microsomal lipid peroxidation systems were evaluated. Organic hydroperoxides react directly with cytochrome P450 to produce reactive oxygenated complexes which cause peroxidation of membrane lipids [24]. CCl_4 is oxidized by cytochrome P450, especially P4502E1 [10], to the trichloromethyl radical, which can subsequently cause lipid peroxidation. Boldine was an effective inhibitor of lipid peroxidation initiated by either t-BHP or CCl_4, with 50% inhibition occurring at boldine concentrations of about 0.015 mM (Table 5).

Microsomal lipid peroxidation was also assayed by determining the increase in oxygen consumption which occurs during active peroxidation in the presence of an iron catalyst. In the absence of ferric–

Table 5. Effect of boldine on iron-independent microsomal lipid peroxidation

Concentration of boldine (mM)	Microsomal lipid peroxidation (nmol/min/mg protein)	
	t-BHP	CCl_4
0	0.61	2.27
0.005	0.36 (41)	1.34 (41)
0.010	0.34 (44)	1.21 (47)
0.015	0.32 (48)	1.11 (51)
0.020	0.23 (62)	1.05 (54)
0.030	0.21 (66)	0.68 (70)
0.050	0.19 (69)	0.45 (80)

Production of TBA-reactive material by rat liver microsomes was determined either in the presence of 3 mM *t*-butyl hydroperoxide (t-BHP) or 2 mM CCl_4 plus 0.8 mM NADPH and the indicated concentrations of boldine. Numbers in parentheses refer to percent inhibition by boldine.

ATP, oxygen uptake with NADPH as the microsomal reductant was about 15 natom/min/mg microsomal protein. The addition of 0.05 mM ferric–ATP increased this rate about 15-fold (Table 6). Boldine had no effect on the NADPH-dependent oxygen consumption in the absence of ferric–ATP (data not shown), but produced a concentration-dependent inhibition of the elevated rates of oxygen uptake found in the presence of ferric–ATP (Table 6). In the absence of ferric–ATP, rates of NADH-dependent oxygen uptake were low and variable; in the presence of ferric–ATP, the rate of oxygen uptake was strikingly elevated to a value one-half that found in the presence of NADPH plus ferric–ATP. Boldine produced a concentration-dependent inhibition of the ferric–ATP catalyzed rates of NADH-dependent oxygen uptake (Table 6). Boldine concentrations of 10–20 μM produced about 50% inhibition of the elevated rates of oxygen uptake, analogous to the inhibitory effectiveness of boldine on the production of TBA-reactive material.

Protection of microsomal oxidative reactions by boldine. Lipid peroxidation is associated with the production of reactive intermediates which are toxic and inhibitory against various enzymes. During microsomal lipid peroxidation, microsomal enzymes such as NADPH–cytochrome P450 reductase, cytochrome P450 and glucose-6-phosphatase are inactivated. The ability of boldine to protect the reductase and cytochrome P450 against lipid peroxidation-mediated toxicity was evaluated. Microsomes were preincubated for 10 min with NADPH in the absence or presence of ferric-ATP and in the absence or presence of boldine. The microsomes were subsequently incubated with DMN or ethanol to assess cytochrome P450-catalyzed mixed-function oxidase activity, or with KMB to assess reductase plus ferric–EDTA catalyzed generation of ·OH-like species. When microsomes were preincubated with NADPH in the absence of ferric–ATP, rates of oxidation of KMB, DMN, or ethanol were similar to rates observed with microsomes not subjected to the preincubation (Table 7). However, when ferric–ATP was present during the preincubation period, the rates of oxidation of all three substrates were decreased, suggesting that the active peroxidation of microsomal lipids led to inhibition of the reductase and cytochrome P450-catalyzed function. The presence of boldine during the preincubation period protected the microsomes against the lipid peroxidation inactivation of the oxidation of all three substrates (Table 7). Almost complete protection could be elicited by boldine concentrations of 0.02 mM.

DISCUSSION

Boldo leaves containing boldine have long been

Table 6. Effect of boldine on microsomal oxygen uptake associated with lipid peroxidation

Concentration of boldine (mM)	Rate of ferric–ATP-dependent oxygen uptake (natom/min/mg protein)	
	NADPH	NADH
0	224	119
0.01	144 (36)	63 (47)
0.02	112 (50)	52 (56)
0.03	62 (72)	35 (71)
0.10	24 (89)	14 (88)

Oxygen uptake was assayed using a Clark electrode in a 3-mL reaction system containing 50 mM Tris, pH 7.4, either 0.5 (NADPH) or 1 (NADH) mg microsomal protein, 50 μM ferric–ATP, and either 0.8 mM NADPH or NADH, and the indicated concentrations of boldine. In the absence of ferric–ATP, the rate of NADPH-dependent oxygen uptake was about 15 natom/min/mg protein, while the NADH-dependent rate was very low (less than 5 natom/min/mg protein). Numbers in parentheses refer to the percent inhibition by boldine. Boldine had no effect on the NADPH- or NADH-dependent rates of oxygen uptake in the absence of ferric–ATP.

Table 7. Effect of boldine on inhibition of microsomal oxidation reactions by lipid peroxidation

Reaction condition	Concentration of boldine (mM)	Rate of oxidation (nmol/min/mg protein)		
		KMB	DMN	Ethanol
No preincubation	0	16.2	6.9	12.4
Preincubation; no FeATP	0	14.6	6.6	11.1
Preincubation; FeATP	0	2.3	3.4	7.5
Preincubation; FeATP	0.02	12.0	6.0	10.6
Preincubation; FeATP	0.03	11.7	8.0	10.7
Preincubation; FeATP	0.10	12.7	7.4	11.0

Rat liver microsomes were preincubated at 37° with NADPH for 10 min in the absence or presence of ferric–ATP, and in the absence or presence of the indicated concentrations of boldine. Tubes were placed on ice and then the following additions were made: KMB plus azide plus ferric–EDTA for the KMB oxidation reaction; DMN; ethanol plus azide. Additional NADPH (0.5 mM) was also added, and the tubes were placed in the 37° incubator for 10 (KMB, ethanol) or 20 (DMN) min. No preincubation refers to tubes kept on ice during the 10-min preincubation period. For the KMB and DMN experiments, Tris buffer was used, whereas for the ethanol experiment, phosphate buffer was used since Tris binds acetaldehyde and lowers the recovery of standard amounts of acetaldehyde.

used for their choleretic, diuretic, sedative and digestive stimulant properties [2]. Boldine has been shown recently to exhibit a strong anti-oxidant activity [4, 5]. The current report demonstrates that boldine is an effective inhibitor of microsomal lipid peroxidation under a variety of conditions. The alkaloid prevents iron-dependent as well as iron-independent lipid peroxidation; enzymatic as well as non-enzymatic (e.g. the ferrous ammonium sulfate system) lipid peroxidation; lipid peroxidation dependent upon electron transfer from two cofactors, and peroxidation independent of electron transfer from NADPH–cytochrome P450 or NADH–cytochrome b_5 reductases (e.g. the t-BHP system); and lipid peroxidation independent of mixed-function oxidase activity of cytochrome P450 (ferric–ATP + NADPH or NADH) as well as that dependent on P450-catalyzed activity (CCl_4 system). Boldine inhibited lipid peroxidation as assessed by two methods: production of TBA-reactive material and oxygen consumption.

The mechanism of action of boldine as a chain-breaking anti-oxidant will require further studies. Boldine did not efficiently interact with superoxide or H_2O_2; therefore, inhibition of lipid peroxidation is not due to scavenging of these reactive oxygen intermediates. Boldine is an effective scavenger of ·OH, equipotent as DMSO. However, under these reaction conditions, microsomal lipid peroxidation is insensitive to superoxide dismutase, catalase and ·OH scavengers [17, 23], indicating that it is independent of superoxide, H_2O_2 and ·OH. Boldine did not inhibit lipid peroxidation by actions on enzymes such as the reductase or P450 or by diverting electrons away from the peroxidative process. Whether boldine itself reacts with lipid radicals produced during the microsomal lipid peroxidation cascade or whether boldine's anti-oxidant action requires or is enhanced by α-tocopherol and the boldine serves to repair the chromanoxyl radical vitamin E as suggested for glutathione, 5-hydroxytryptamine and 5-hydroxyindole [25, 26] is not known. That boldine has a direct anti-oxidant action on its own is indicated by the prevention of alkylperoxyl radical inactivation of lysozyme by the alkaloid in a chemical system devoid of vitamine E [4].

Many anti-oxidants, natural and synthetic, have been found to be effective inhibitors of microsomal lipid peroxidation. Some special features of the anti-oxidant activity of boldine which distinguish the alkaloid from certain other anti-oxidants include the following. Certain anti-oxidants such as propylgallate [27] or phytic acid [28] can also act as iron chelators and enhance auto-oxidation of ferrous iron to the ferric state [29]; the inhibition of lipid peroxidation by phytic acid can be overcome by increasing the concentration of iron [30]. Boldine depresses iron-independent lipid peroxidation, and is effective at concentrations (e.g. 5–15 μM) below the added iron concentration employed (50–100 μM) to catalyze lipid peroxidation. Some synthetic anti-oxidants such as butylated hydroxyanisole, cyanidanol-3, ethoxyquin, and propylgallate have been shown to increase rat liver microsomal production of H_2O_2 [31–33]; many of these anti-oxidants, as well as butylated hydroxytoluene decrease cytochrome P450 content and depress mixed-function oxidase activity of microsomes [34–37]. The anti-oxidant activity associated with these anti-oxidants appears to enhance the oxidase function of cytochrome P450. Boldine did not increase (or decrease) the production of H_2O_2 by microsomes in the absence or presence of ferric–EDTA, suggesting that it was not a substrate for redox cycling by NADPH–P450 reductase nor did it elevate oxidase activity of P450. Boldine did not suppress mixed-function oxidase activity of the microsomes as it had no effect on oxidation of ethanol or dimethylnitrosamine. The latter also indicates that boldine does not divert reducing

equivalents from NADPH away from the P450 cycle as do anti-oxidants such as *o*-naphthoquinones (e.g. β-lapachone) [38] or *p*-quinones such as menadione [39, 40], which inhibit P450 mixed-function oxidase activity. Moreover, *o*-naphthoquinones and *p*-quinones because of their diverting electrons for their own redox cycling activity are not effective in inhibiting organic hydroperoxide-dependent microsomal lipid peroxidation [38, 40]. Boldine was as effective in inhibiting organic hydroperoxide-dependent lipid peroxidation as it was in decreasing NADPH or NADH plus iron-dependent lipid peroxidation. Anti-oxidants such as propylgallate, butylated hydroxyanisole or toluene, and vanillin have been reported to stimulate ·OH production by Fenton-type systems [41–44]. Boldine did not stimulate ·OH production by either the microsomes or xanthine oxidase reaction systems.

Unlike many of the above-mentioned anti-oxidants, boldine did not inhibit hepatic mixed-function oxidase activity. Boldine was very effective in preserving reductase and P450 catalytic activity against inactivation by the lipid peroxidation process. Control rates of oxidation of KMB (reductase-mediated in the presence of ferric–EDTA) or of DMN and ethanol (effective substrates for cytochrome P4502E1) were maintained when boldine was present during a preincubation period in which active lipid peroxidation reduced these activities when boldine was not present. Many of the above features, including the very low toxicity associated with boldine (e.g. anti-oxidants such as butylated hydroxytoluene and nordihydroguaiaretic acid are banned) make this alkaloid an attractive candidate for further evaluation as a clinically useful anti-oxidant.

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