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Inactivation of Liver Alcohol Dehydrogenases and Inhibition of Ethanol Metabolism by Ambivalent Active-Site-Directed Reagents

Wen-Sherng Chen, David P. Bohlken, and Bryce V. Plapp*

Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242. *Received May 1, 1980*

Active-site-directed reagents, of the general structure ω-($BrCH_2CONH$)RCOY, where R = alkyl, aryl, or aralkyl, and Y = OH or NH_2, inactivated horse, mouse, rat, and human liver alcohol dehydrogenases at widely different rates, reflecting differences in reagent specificity and in the structures of the enzymes. Treatment of mice and rats with either of two optimally specific reagents, p-(XCH_2CONH)$C_6H_4(CH_2)_3CONH_2$, where X = Br (7) or CH_3SO_3 (10), partially (20 to 40%) inactivated alcohol dehydrogenase in liver, inhibited ethanol metabolism, and prolonged the impairment of coordination produced by ethanol in these animals. Although the dose of 7 used (0.13 mmol/kg) approximated the LD_{50}, 10 was effective at a dose of 0.48 mmol/kg that was not acutely toxic.

The first step, and one of the slowest steps, in the metabolism of alcohols in animals is catalyzed by liver alcohol dehydrogenase (EC 1.1.1.1). Thus, this enzyme would be a rational target for inhibiting alcohol metabolism and thereby for therapeutically preventing poisoning by methanol or ethylene glycol or for experimentally studying the potentially deleterious effects of ethanol metabolism. Pyrazole and some of its 4-substituted derivatives are especially strong inhibitors of the enzyme and inhibit ethanol metabolism, but pyrazole is itself toxic and may not be useful for treatment of humans.[1] Thus, we are trying to develop inhibitors.

Based on a knowledge of the three-dimensional structure of the horse liver enzyme, we designed some ω-(bromoacetamido) fatty acids and analogues that are "ambivalent" active-site-directed inactivators of the enzyme.[2] The reagents can bind to and inactivate the enzyme in two different ways. By binding into the coenzyme (NAD) binding site[3] of the enzyme not complexed with coenzyme, a cysteine residue is alkylated. In the enzyme complexed with coenzyme, the cysteine is protected, and the reagent binds in the substrate binding pocket and reacts—in a facilitated manner—with a methionine residue. In order to develop reagents that would be efficacious in vivo, we have compared the reactivities of varied reagents with the enzymes from different species, prepared some new analogues with functional groups of potential utility in vivo, and evaluated the reagents in experimental animals.

Results and Discussion

The ω-(bromoacetamido) fatty acids were designed to bind with their carboxyl groups ligated to the active-site zinc ion in the enzyme–NAD^+ complex and bridge to alkylate methionine residue 306. In the previous work with the horse enzyme, reagents about 14 Å long were of optimal length to fit into the pocket and give the best facilitation of inactivation. Since the rat and human liver enzymes also have methionine-306,[3] the reagents should also inactivate these enzymes, even though we should expect that the active sites of enzymes from different species would differ somewhat and thus give rates of inactivation that varied with the structure of the reagent. The results in Table I bear out this expectation and can be explained as follows.

In the absence of NAD^+, the reagents can inactivate the horse enzyme by reacting with a cysteine residue[2] in the active site. As the size and hydrophobicity of the reagent increase, the rate of inactivation increases until the rate is much faster than that found for the simple, nonspecific reagents, $BrCH_2COOH$ and $BrCH_2CONH_2$, which also react with cysteine. In the presence of NAD^+, the reaction of cysteine is generally greatly inhibited, but 2 and 6 inactivate more rapidly than in the absence of NAD^+ and react with a methionine residue. These two reagents apparently fit nicely into the active site in the presence of NAD^+. The rat enzyme shows a similar pattern of inactivation, even though the absolute rates differ, and 3 and 6 give the most facilitation in the presence of NAD^+. The mouse enzyme is generally less reactive, but 6 still inactivates most rapidly in the presence of NAD^+. If the mouse enzyme has methionine-306, it is possible that its sulfur is less accessible than in the other species. The human enzyme gives a very different pattern, with 2–4 and 6–9 showing considerable facilitation by NAD^+. In this case, the methionine may be more exposed or the active site may be more flexible so that longer (3 and 4) or more rigid (8 and 9) reagents can bind in a productive manner.

Although the size and shape of the reagent is probably not the best that can be obtained, the results in Table I indicate that 6 has the most promising affinity group for in vivo evaluation in mice and rats. On the other hand, the reagent should probably be modified in two ways for better efficacy in vivo. First, the carboxyl groups should be amidated, since fatty acid amides bind tightly to the enzyme–NADH complex,[4] which may be a significant form of enzyme in vivo. Amides can also bind less tightly to the enzyme–NAD^+ complex[5] and give facilitated inactivation.[2] Moreover, for use as an agent to inhibit alcohol metabolism, it should be noted that amides give uncompetitive inhibition vs. alcohol, and therefore high alcohol concentrations do not prevent inhibition. Secondly, the bromoacetyl group may react too fast with SH compounds in the animal to allow the reagent to get into the liver and react with the dehydrogenase. Thus, the mesyl (10) and chloro (11) analogues were prepared and evaluated on the purified enzymes.

Table II shows that the reactivities of the carboxylic acid (6) with the various enzymes are reduced in the presence of NADH, whereas the reactivities of the amide (7) are facilitated by NADH and by NAD^+, as expected. The mesyl compound (10) reacts very slowly with the horse enzyme in the absence of nucleotides, and about one-tenth as fast as the bromo compound (7) in the presence of NAD^+ or NADH. (10 also reacts by an active-site-directed mechanism,[2] binding with a dissociation constant of 120 μM to the enzyme–NADH complex and being converted to the irreversibly inactivated enzyme with an unimolecular rate constant of 0.0017 min^{-1}). The chloro compound (11)

(1) B. V. Plapp, "Biochemical Pharmacology of Ethanol", E. Majchrowicz, Ed.; Plenum Press, New York, 1975, pp 77–109.
(2) W.-S. Chen and B. V. Plapp, *Biochemistry*, 17, 4916 (1978).
(3) C.-I. Brändén, H. Jörnvall, H. Eklund, and B. Furugren, *Enzymes, 3rd Ed.*, 11, 103 (1975).
(4) A. D. Winer and H. Theorell, *Acta Chem. Scand.*, 14, 1729 (1960).
(5) D. S. Sigman and A. D. Winer, *Biochim. Biophys. Acta.*, 206, 183 (1970).

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Table I. Specificity of Inactivation of Liver Alcohol Dehydrogenases in Vitro by Bromoacetamidocarboxylic Acids in the Presence or Absence of NAD^+

		apparent 2nd order rate constants, $M^{-1}\cdot min^{-1}$							
		horse[a]		rat		mouse		human	
no.	compound	no addn	+1mM NAD^+	no addn	+1mM NAD^+	no addn	+1mM NAD^+	no addn	+1mM NAD^+
1	$BrCH_2CONH(CH_2)_6COOH$	5.9	0.5	11	0.8	7.6	b	6.7	8.3
2	$BrCH_2CONH(CH_2)_7COOH$	6.3	7.2	12	4.0	4.9	b	6.7	50
3	$BrCH_2CONH(CH_2)_8COOH$	10	4.9	12	12	4.9	b	23	240
4	$BrCH_2CONH(CH_2)_{10}COOH$	43	3.0	9.1	4.0	8.8	b	230	1400
5	$BrCH_2CONH(CH_2)_{11}COOH$	120	3.2	19	1.0	43	b	140	320
6	p-$(BrCH_2CONH)C_6H_4(CH_2)_3COOH$	100	120	25	32	38	14	11	140
8	4-[p-$(BrCH_2CONH)C_6H_4$]C_6H_4COOH	140	24	29	25	43	6.3	50	3500
9	4-[p-$(BrCH_2CONH)C_6H_4O$]C_6H_4COOH	320	17	69	23	173	11	26	290
	$BrCH_2COOH$	8.3	0.09	13	0.8	9.8	b	0.8	0.8
	$BrCH_2CONH_2$	1.6	0.01	5.3	b	8.8	b	2.9	2.9

[a] Constants for inactivation of the horse enzyme were obtained previously.[2] [b] Inactivation was so slow that the enzyme activity was the same as the control after 1 day of reaction.

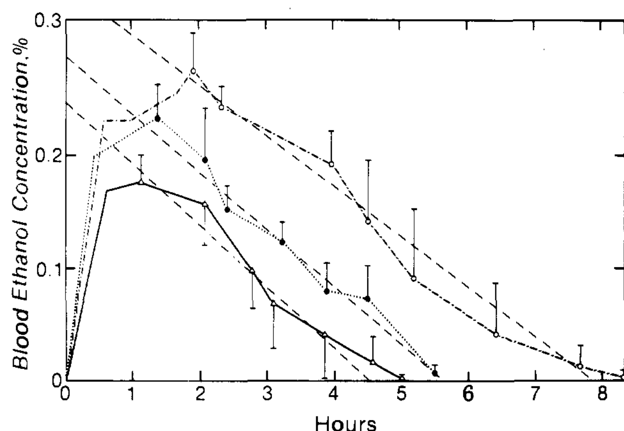

Figure 1. Inhibition of ethanol metabolism in mice. Groups of four mice were treated with 0.48 mmol/kg of **10** (○) or 0.13 mmol/kg of **7** (●) or with sterile saline (△) 22–23 h before being given doses of 3 g of ethanol/kg at zero time. Each point represents the mean blood ethanol concentration for four mice, with the vertical lines indicating SD. The dashed lines are weighted, linear least-squares fits of the means, not including point obtained during the absorption of ethanol (1–2 h). Although the rate of disappearance of ethanol from the blood is given by the slope of the blood alcohol curve, the rate of *metabolism* of ethanol should be calculated by multiplying the rate of decrease (slope, β value) by the ratio (r) of the dose (mol/kg) to the theoretical blood level at zero time (mol/kg).[7] This calculation of the rate of metabolism is mathematically equivalent to dividing the dose by the time required to eliminate all of the ethanol. The calculation of r corrects for variations in the distribution of ethanol in the "body water", which depends upon the species, sex, and pharmacologic and physiologic state of the animal. The causes of the variability of r are not well understood, but the lower values of r obtained for the treated mice may arise simply because inhibition of ethanol metabolism allows more complete equilibration of ethanol in the blood. The differences in the r values in this figure are not due to differences in techniques, since the experiments were conducted on consecutive days (or on the same day for the control and mice treated with **7**) by the same persons using the same procedures.

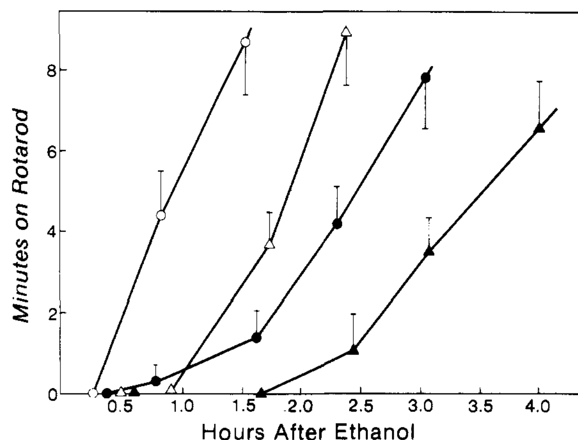

Figure 2. Inhibition of recovery of rotarod performance in mice. Groups of five mice were treated with 0 mmol/kg (○), 0.13 mmol/kg (△), 0.24 mmol/kg (●), or 0.48 mmol/kg (▲) of **10** 23 h before administration of 3 g of ethanol/kg at zero time. Each point represents the mean time the mice can stay on the rotarod, and the vertical lines indicate SD.

was about one-tenth as reactive as **10** and judged to be too unreactive to be of use in vivo. On the other hand, such carboxamides can also "inactivate" the enzyme by formation of a slowly dissociating enzyme–NAD^+–amide complex. An indication of the rapidity of formation of this complex was obtained from a study of the "inactivation" by **12**, which cannot alkylate the methionine but which led to 74% inhibition of enzyme under the conditions used. Although this inhibition is apparently reversible, it could produce inhibition of alcohol metabolism. Thus, **6**, **7**, **10**, and **12** were chosen for in vivo studies.

Mice and rats were injected intraperitoneally with a single dose of reagent 22–24 h before ethanol was administered. The treatment period should be long enough to allow the reagents to inactivate most of the alcohol dehydrogenase, as estimated from the kinetics of inactivation in vitro, but not so long as to allow synthesis of new enzymes. (The half-life for turnover of the enzyme in rats is about 4.9 days.[6]) As shown in Figure 1, pretreatment of mice with **7** or **10** significantly inhibited ethanol metabolism as measured from the (computed) time required to eliminate the total amount of ethanol.[7] Similar experiments were performed with the other compounds and with rats.

For confirmation of the effects of the reagents on ethanol metabolism and as a test of their side effects, the ability of mice to walk on a rotating rod was determined. Mice that had been treated with reagent for 22–24 h were indistinguishable from control mice, but, as shown in Figure 2, none of the mice treated with ethanol could hang onto the rotarod during the first 15 min. At about 75 min after receiving the ethanol, the average time that untreated mice could hang onto the rod was 7.5 min, which was defined as 50% impairment of rotarod performance. Treatment

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(7) H. Kalant, "The Biology of Alcoholism", Vol. I, B. Kissin and H. Begleiter, Eds., Plenum Press, New York, 1971, pp 1–62.

Table II. Effects of Reagent Structures and Coenzymes on the Inactivation of Liver Alcohol Dehydrogenases in Vitro

p-$(XCH_2CONH)C_6H_4(CH_2)_3COY$

			apparent 2nd order rate constants, $M^{-1} \cdot min^{-1}$											
			horse			rat			mouse			human		
no.	X	Y	no addn	1 mM NAD^+	0.2 mM NADH	no addn	1 mM NAD^+	2 mM NADH	no addn	1 mM NAD^+	0.2 mM NADH	no addn	1 mM NAD^+	0.2 mM NADH
6	Br	OH	100	120	15	25	32	15	40	13	8	11	140	5.1
7	Br	NH_2	40	190[a]	160	35	70[a]	35	36	60[a]	29	5.4	320[a]	5.4
10	CH_3SO_3	NH_2	*b*	24[a]	13									
11	Cl	NH_2	*b*	2.5[a]	1.6									
12	H	NH_2		1500[c]										

[a] These amides inactivated with biphasic kinetics in the presence of NAD^+; the fast phase is apparently due to formation of a slowly dissociable enzyme-NAD-amide adduct, and the slow phase is presumably due to alkylation of the enzyme. The numbers reported above are for the slow phase. The human enzyme showed only one phase. [b] Inactivation was so slow that the enzyme activity was the same as the control after 1 day of reaction. [c] The rate constant corresponds to the only phase of inhibition.

Table III. Evaluation of Inactivators in Vivo[a]

		% inhibition						
		rate of ethanol metabolism[b]		ADH act. in liver[c]		recovery of rotarod performance	LD_{50}, mmol/kg	
compd	dose, mmol/kg	mice	rats	mice	rats	mice	mice	rats
6	0.13		24		27			
7	0.13	20	26	21	21	58	0.21[d]	0.13[e]
10	0.48	43	15	38	35	70[f]	>0.48[g]	>0.48[g]
12	0.48	60	0	43	8	60	>0.48[g]	>0.48[g]
$BrCH_2CONH_2$	0.13	18	11	27	0	28	(0.27–1.1)[h]	

[a] Four or five mice or two rats were used in each experiment. [b] The rate of metabolism of ethanol in seven control mice was 14 ± 2 mmol h^{-1} (kg of body weight)$^{-1}$, and in four control rats it was 9.7 ± 0.6 mmol h^{-1} (kg of body weight)$^{-1}$. [c] The specific activity of the enzyme in five control mice was 0.0029 ± 0.006 units/mg of protein and in four rats it was 0.0073 ± 0.0007 units/mg of protein. [d] The lower and upper limits of the LD_{50} are 0.16 mmol/kg and 0.28 mmol/kg at $p \leq 0.05$ for three doses on 15 mice. [e] Estimated lethal dose: one of two rats died. [f] The ED_{50} of the inhibition of recovery of rotarod performance was 0.24 (0.13–0.45) mmol/kg at $p \leq 0.1$ for three doses of the compound on 20 mice. [g] The animals survived at this dose of compound. [h] The LD_{50} is probably within the range shown, since the LD_{50} for $ClCH_2CONH_2$ is 1.1 mmol/kg and the LD_{50} for ICH_2CONH_2 is 0.27 mmol/kg.[9]

with the reagents prolonged the impairment of performance in a dose-dependent manner. By inhibiting ethanol metabolism, it appears, the reagents prolong the effect of ethanol on coordination.

The pharmacological evaluations presented in Table III permit several diverse conclusions. Most general, the alkylating amides (**7** and **10**) consistently and significantly inhibited ethanol metabolism, inactivated alcohol dehydrogenase in liver, and prolonged the impairment of coordination produced by ethanol. More particularly, it is noted that the bromoacetamido compound (**7**) was not very efficacious, since the dose used approached the LD_{50}. In contrast, the mesyl compound (**10**) was more effective, at a sublethal dose, in mice but not in rats. The lower toxicity of the mesyl compound justifies its use in vivo in preference to the bromo compound. In mice, however, **12** (which is not an alkylator) was just as effective as **10**. This result probably is due to tight, reversible inhibition of alcohol dehydrogenase in mice and indicates that alkylation is not a prerequisite for effectiveness in mice. In rats, on the other hand, **12** was not effective, even though 0.7 mmol/kg of n-butyramide can inhibit ethanol metabolism by 50% in rats.[8] Thus, alkylating ability (as with **7** and **10**) seems to be required in rats and can even be expressed with **6**, which has a carboxyl group rather than the carboxamide. Nevertheless, a simple alkylator, $BrCH_2CONH_2$, was not effective in rats, even though it may have some nonspecific effects in mice. Overall, these results suggest that the requirements for effectiveness of compounds in mice and rats differ and should be taken into account in the design of reagents for these animals or for humans.

Another general observation is that these compounds did not completely inactivate alcohol dehydrogenase in vivo or completely inhibit ethanol metabolism. Based on the in vitro results, we expected that **10** would inactivate the enzyme with a half-time of about 8 h so that after 22 h the enzyme would be inactivated by at least 80%. (**7** should act faster.) Possible explanations for the small effects are that the compounds are being inactivated by metabolism or that the enzymes are being protected by some endogenous materials (such as fatty acids) that bind to the active site.

In conclusion, it appears that we can design active-site-directed inactivators of alcohol dehydrogenase that are at least somewhat efficacious in vivo. A demonstration that the reagents specifically inactivate the enzyme in vivo, however, is confounded by the effects of reversible inhibition and nonspecific alkylation. In order to develop more effective compounds, we expect that the affinity group (e.g., phenylbutyryl) must be improved so that binding is more specific and so that reactivity is enhanced by better juxtaposition of the reactive functional group and the nucleophile on the enzyme. It may also be necessary to develop nonalkylating functional groups (directed toward amino acid residues other than methionine) if nonspecific reactions and chronic toxicity are to be avoided. (Although

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(9) "Registry of Toxic Effects of Chemical Substances", U.S. Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, NIOSH, Cincinnati, OH, 1978.

the bromoacetamido reagents react selectively with a methionine residue of liver alcohol dehydrogenase complexed to coenzyme, the reagents can react at similar rates with sulfhydryl groups, as indicated by the results of kinetic and chemical studies on the enzyme not complexed with coenzyme.[2]) We think the results confirm that carboxamides are quite suitable groups for inducing selective binding to alcohol dehydrogenase, since the amides bind to complexes with NAD^+ or NADH, and that the amides are useful in vivo.

Experimental Section

Chemistry. The synthetic procedures, intermediate compounds, and other methods have been described previously.[2] The following new compounds were prepared similarly.

4-(*p*-Mesylglycolamidophenyl)butyramide (10). Mesylglycolic acid was prepared by adding an equivalent of ICH_2COOH to a solution of CH_3SO_3Ag in CH_3CN and stirring until precipitation of AgI ceased. AgI was removed by filtration and the filtrate was evaporated to dryness. The mesylglycolic acid was extracted with diethyl ether and recrystallized from acetone–$CHCl_3$-petroleum ether in 50% yield, mp 111–114 °C. Mesylglycolic acid was coupled to *N*-hydroxysuccinimide with *N,N'*-dicyclohexylcarbodiimide (equivalent amounts of each reagent) in THF with stirring at 4 °C for 20 h. *N,N'*-Dicyclohexylurea was removed by filtration, and the dried filtrate was recrystallized from CH_3CN–$CHCl_3$–petroleum ether (1:1:5) with a 56% yield, mp 115–117 °C. A suspension of 4-(*p*-aminophenyl)butyramide in CH_3CN was acylated by an equivalent amount of *N*-[(mesylglycolyl)oxy]succinimide for 2 days at room temperature. The solvent was evaporated, and the crude product was washed with H_2O, dissolved in DMF, and purified on a column of silica gel developed with $CHCl_3$–CH_3OH–CH_3COOH (450:50:1). The product (10) was recrystallized from DMF–$CHCl_3$–petroleum ether (1:1:5) in 63% yield, mp 138–140 °C. Anal. ($C_{13}H_{18}N_2O_5S$) C, H, N.

4-(*p*-Chloroacetamidophenyl)butyramide (11). 4-(*p*-Aminophenyl)butyramide was acylated with 1 equiv of $ClCH_2COCl$ in CH_3CN at 25 °C for 48 h. The product was purified on silica gel as for 10 and crystallized from $CHCl_3$–CH_3OH–petroleum ether (1:1:5) in 46% yield, mp 146–148 °C. Anal. ($C_{12}H_{15}N_2O_2Cl$) C, H, N.

4-(*p*-Acetamidophenyl)butyramide (12). 4-(*p*-Aminophenyl)butyramide was suspended in a 20-fold excess of $(CH_3CO)_2O$ at room temperature for 20 min. The product was precipitated with petroleum ether and recrystallized from THF–petroleum ether in 70% yield, mp 155–157 °C. Anal. ($C_{12}H_{16}N_2O_2$) C, H, N.

Enzymology. Crystalline EE isozyme of alcohol dehydrogenase was prepared from horse liver.[10] Enzymes from mouse, rat, and human livers were partially purified in order to remove interfering NADH-oxidase and NAD^+-reductase activities. Mouse enzyme was purified fourfold by precipitation with $(NH_4)_2SO_4$ (45–65% saturation) and chromatography on diethylaminoethylcellulose (DEAE-cellulose).[11] Rat enzyme was purified 15-fold by precipitation with $(NH_4)_2SO_4$ (35–75% saturation) and chromatography on DEAE-cellulose.[12] The human enzyme was prepared by chromatography on carboxymethylcellulose and DEAE-cellulose and by precipitation with $(NH_4)_2SO_4$ (60% saturation) as described,[13] except that 5% ethanol was added throughout the procedure to stabilize the enzyme. The ethanol was then removed by dialysis before the enzyme was used for inactivation studies.

Rates of inactivation of purified dehydrogenases in 0.1 M $Na_4P_2O_7$ buffer adjusted to pH 8 with H_3PO_4 were determined at 25 °C with 0.2 mM reagent. This concentration of reagent approximates that used in vivo. For comparative purposes, the apparent second-order rate constant for inactivation was calculated on the assumption that the reaction was first order in reagent. The effects of coenzyme on inactivation were tested by adding NAD^+ or NADH to the enzyme (about 0.2 mg/mL) 1 min before inactivator was added.

For determination of alcohol dehydrogenase activity in livers from treated mice or rats, the liver was removed just after the blood ethanol concentration approximated zero, chilled on crushed ice, and homogenized. The homogenate was clarified by centrifugation, and the alcohol dehydrogenase activity was determined from the difference in initial velocities in an assay[14] with ethanol and NAD^+ and in a control assay containing 10 mM pyrazole, which specifically inhibits alcohol dehydrogenase. Protein content was determined and the specific enzymatic activity was calculated.

Pharmacology. Fed male Swiss–Webster mice (20–25 g) and male Sprague–Dawley rats (160–200 g) were used. The compounds were thoroughly suspended in sterile 0.9% saline by using a Potter–Elvejhm tissue grinder 30 min prior to intraperitoneal injection. Ethanol doses of 3 g/kg for mice and 2 g/kg for rats, as a 40% (w/v) solution in saline, were administered intraperitoneally 22–24 h after the reagent was given. Blood ethanol levels were measured on a Beckman GC 72-5 gas chromatograph with a flame-ionization detector and a column ($^1/_8$ in. × 6 ft) of Porapak S, 50–80 mesh. The operating conditions were: column, 150 °C; column detector, 275 °C; inlet, 275 °C; detector line, 212 °C; flow rate of carrier gas (N_2), 50 mL/min; flow rate of H_2, 45 mL/min; flow rate of air, 250 mL/min. The column was preconditioned at 150 °C overnight with full carrier gas flow.

A modified method[15] was used to prepare blood samples for gas chromatography. A 5 μL sample of blood was periodically withdrawn from tail veins of mice or rats and added to a 0.5-mL polypropylene centrifuge tube containing 50 μL of 2.4 mM 1-propanol (as an internal standard) and 20 μL of 0.14 M $Ba(OH)_2$. After about 5 min, 20 μL of 0.14 M $ZnSO_4$ was added and the mixture was centrifuged for 4 min at 10000*g* and 4 °C. About 3 μL of the supernate was injected into the gas chromatograph.

Rotarod performance[16] was used to test for effects on the central nervous system. Five mice were used per dose level of compound. The mice were trained to hang onto a 2-cm wooden rod rotating at 7 rpm for two consecutive periods of 15 min with 15 min of rest in between. Five vehicle-treated mice were used as controls. The reagents were injected intraperitoneally 22 h prior to administration of ethanol. The percentage of impairment of rotarod performance was calculated from the following formula: 100(15 – minutes on the rod)/15. The percentages were transformed to probits with a probit transformation table. $t_{1/2}$, the time required to regain 50% of the normal rotarod performance, was calculated by regression analysis. ED_{50} was obtained from the log dose–response plot as usual, where the response was calculated from $[t_{1/2}(\text{treated}) - t_{1/2}(\text{control})]/t_{1/2}(\text{treated})$.

LD_{50}'s refer to acute toxicities in a 24-h period after a single dose of reagent.

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