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NARCAN® INHIBITION OF HUMAN LIVER ALCOHOL DEHYDROGENASE

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Narcan®, the pharmaceutical agent for the administration of naloxone, has been reported to antagonize ethanol intoxication. In addition to naloxone, Narcan® contains the antioxidant esters methyl- and propylparaben. Pure naloxone and these two esters were examined for their capacity to inhibit ethanol oxidation by purified isozymes of human liver alcohol dehydrogenase (ADH). Naloxone (400 micromolar) fails completely to inactivate any of the three ADH isozyme classes. In contrast, methyl- and propylparaben, and some related esters, competitively inhibit the oxidation of ethanol and reduction of acetaldehyde by all isozymes examined. The reported effects of Narcan® on ethanol-intoxicated animals or cells cannot be attributed to the action of naloxone.

Naloxone has been reported to be an antagonist of narcotics and useful in the management of overdoses of opiates. It is further said to counteract the effects of ethanol intoxication in rodents and man (1-5). Doses from 0.2 to 2.0 mg of naloxone have been reported to restore consciousness in ethanol induced coma (2). Although some of these reports are not explicit, naloxone must be presumed to have been administered in the only form that is commercially obtainable for therapeutic purposes i.e., Narcan®, which contains methyl- and propylparaben (p-hydroxybenzoate methyl and n-propyl esters). The results have been interpreted to imply similarities both in the metabolism and/or mechanism of action of ethanol and opiates (6). However, conflicting reports suggest that the drug neither affects the metabolism nor the consequences of ethanol comsumption (7-10). Thus, both the efficacy and

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Abbreviations: ADH, alcohol dehydrogenase; NAD^+, β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; propylparaben, propyl p-hydroxybenzoate; methylparaben, methyl p-hydroxybenzoate; naloxone, 17-allyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one.

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dosage of Narcan® (naloxone) as an ethanol antagonist remain to be established.

We have examined the effect of Narcan® on ethanol oxidation in vitro and found it to inhibit all three classes of human liver alcohol dehydrogenase (ADH; EC 1.1.1.1). However, on further scrutiny this inhibition turns out to be due to the adjuvants methyl- and propylparaben, not to naloxone. Authentic naloxone alone does not effect the catalytic activity of any of the human ADH isozymes. Thus, this agent and the manifestations of ethanol metabolism are unrelated. The study of this problem further led to the demonstration that in addition to the parabens, a series of other esters inhibit ADH isozymes.

Material and Methods

Human liver alcohol dehydrogenase isozymes of Class I ($\beta\gamma_1$, $\beta\gamma_2$, $\alpha\beta$, $\alpha\gamma_1$, $\beta\beta$,) Class II (π) and Class III (χ), prepared and assayed as described previously (11-13), were homogeneous as judged by electrophoresis on both starch (14) and sodium dodecyl sulfate polyacrylamide gels (15) and were identified by urea polyacrylamide gel electrophoresis (16). Protein concentration was measured by the method of Lowry (17) using bovine serum albumin as a standard. Narcan® was obtained from Endo Pharmaceuticals, Puerto Rico; methyl benzoate, ethyl benzoate, ethyl isobutyrate, and propylparaben from Aldrich Chemical Company, Milwaukee, WI; ethyl acetate from Fisher Chemical Co., Springfield, NJ. Chemically authentic naloxone was a gift from E.I. Du Pont de Nemours & Co. Inc., Wilmington, DE. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

ADH activity was measured using ethanol as the substrate and monitoring the production of NADH at 340 nm, $\epsilon = 6220\ M^{-1}\ cm^{-1}$, with a Gilford Model 2600 spectrophotometer. All assays were carried out in glycine (100 mM, pH 10.0) or TES buffer, 50 mM, pH 7.0, with 2.5 mM NAD^+ at 25°C. Assays of the reduction of pentanal were in 50 mM TES buffer pH 7.0, in the presence of 200 µM NADH by methods described previously (18). All solutions of esters and substrates were prepared with deionized glass-distilled water and used within ten hours. Inhibition by methyl-, ethyl- and propylparaben was examined by varying the concentration of ethanol or pentanal while keeping that of the esters constant. Inhibition constants were determined from Lineweaver-Burke plots. In studies of the inhibition of the $\beta\gamma_1$ isozyme by other esters the concentration of substrate was kept constant (0.67 mM for ethanol or 9 µM pentanal), while varying inhibitor concentration. The amount of ester required for 50% inhibition of catalysis was calculated from a plot of concentration of inhibitor vs. percent inhibition.

Results

Naloxone, at concentrations up to 400 µM, is neither an inhibitor nor a substrate of any of the human liver alcohol dehydrogenase isozymes. However, Narcan®, the propyl- and methylparaben-containing pharmaceutical agent for the therapeutic administration of naloxone, is an inhibitor (Table I). The

TABLE I

Narcan®, Propylparaben and Methylparaben Inhibition of Ethanol Oxidation by Human ADH Isozymes[a]

Isozyme	K_i (μM)[b] Narcan®	Propylparaben	Methylparaben
$\beta\gamma_1$	9.7	8.9	140.0
$\alpha\beta$	8.1	9.1	102.5
$\alpha\gamma_1$	11.1	14.3	327.5
$\beta\gamma_2$	4.3	7.3	415.0
$\beta\beta$	61.3	79.0	3040.0
π	7.9	6.5	34.0
χ	>100	300	>3000

[a] Measured at pH 10.0. [b] K_i calculated as described in the text. K_i's for Narcan® are based on the concentration of propylparaben in the solution, which is equal to the concentration of naloxone.

inhibition constants obtained for the $\alpha\gamma_1$, $\beta\gamma_2$, and $\alpha\beta$ isozymes agree closely with that for $\beta\gamma_1$ whereas for all esters the $\beta\beta$ isozyme exhibits K_i values approximately 10 fold greater than the others of Class I. In Narcan®, the concentration of both naloxone and propylparaben is 1.1 mM, while that of methylparaben is 11.8 mM. Both these esters competitively inhibit all the isozymes examined (Table I). The K_i values show that propylparaben itself can account fully for the inhibition by Narcan® *in vitro*.

The $\beta\gamma_1$ isozyme was chosen to extend these studies as representative of other Class I isozymes. At pH 7, methyl- and ethylparaban competively inhibit ethanol oxidation with K_i values of 83 and 12 μM respectively. At this pH, optimal for reduction, they are also competitive with pentanal with K_I's of 220 and 90 μM, respectively.

A variety of additional esters inhibit the $\beta\gamma_1$ isozyme catalyzed oxidation of ethanol both at pH 7 and 10 (Table II). Aromatic esters are more effective than alkyl esters. An increase in the length of the side chain on p-hydroxybenzoate esters improves binding while eliminating the p-hydroxy group weakens it, as in ethyl benzoate. These trends coincide with those found for amide inhibitors of ADH (19).

TABLE II

Ester Inhibition of the $\beta\gamma_1$ Isozyme in Ethanol Oxidation

Inhibitor	pH	$I_{0.5}$ (μM)
methylparaben	10.0	225
	7.0	83
ethylparaben	10.0	46
	7.0	48
propylparaben	10.0	15
	7.0	12
ethyl benzoate	10.0	185
	7.0	160
ethyl nicotinate	10.0	1420
	7.0	330
methyl vanillate	10.0	200
	7.0	130
ethyl acetate	10.0	3000
ethyl isobutyrate	10.0	980

Discussion

Narcan® has been advocated as an antagonist for the manifestations of human ethanol intoxication. The present study of the potential effects of naloxone, its supposed active ingredient, on the isozymes of human liver ADH was undertaken to uncover relationships, if any, of this drug to ethanol metabolism. In point of fact, we did not detect any effect of naloxone on the action of any of the human liver alcohol dehydrogenase isozymes. Pure naloxone is not available commercially, but the pharmaceutical preparation, Narcan®, is available easily. This very specific circumstance led us to determine both that naloxone has no effect on ADH catalysis and that Narcan® contains the anti-oxidant ester adjuvants propyl- and methylparaben that turn out to be inhibitors of the enzyme.

The inhibition constants of propylparaben and methylparaben for the Class I and II isozymes are quite similar, with K_I's ~10 μM in all cases (Table I).

This contrasts with 4-methylpyrazole which inhibits Class I isozymes far better than either Class II and III and serves as a basis for the differentiation of Class I from Class II and III (14). 4-Methylpyrazole, which profoundly effects in vivo ethanol metabolism (13,14), virtually does not affect the Class II and III isozymes at all. Since propylparaben, and to a lesser extent methylparaben, inhibit all three human ADH isozyme classes, they might also be expected to inhibit ethanol oxidation in vivo, though this hypothesis requires experimental examination.

Following the lead suggested by the parabens we have found that a variety of esters inhibit both ethanol oxidation and pentanal reduction, though, thus far, propylparaben is the most effective. From these kinetic studies, which show competitive inhibition with the second substrate in both oxidative and reductive catalysis, we can compare such esters with other ADH inhibitors. Esters do not bind to the enzyme in the same mode as do amides, which are competitive with alcohols but noncompetitive with aldehydes (19-21). Both pyrazoles and esters are competitive with ethanol. Short side-chain 4-substituted pyrazoles, e.g. 4-methylpyrazole, inhibit Class II isozymes four orders of magnitude more effectively than Class I, while short-side chain esters are equally effective inhibitors of both classes. Crystallographic and nuclear magnetic resonance studies of the ternary complex of the horse ADH EE isozyme with NAD^+ and pyrazole show that the inhibitor binds to both the active site zinc atom and carbon-4 of the amide-bearing ring of NAD^+ through the two nitrogen atoms in the pyrazole ring (22,23). By analogy to the mode of binding suggested for amides (19), ester binding likely involves the carbonyl oxygen as a ligand to the metal.

Esters are less effective inhibitors of the $\beta\beta$ isozyme than of other Class I isozymes. In the case of methyl- and propylparaben, the K_I's for $\beta\beta$ are approximately 10-fold greater than for $\beta\gamma_1$, $\alpha\beta$, $\alpha\gamma_1$ and $\beta\gamma_2$. For alkyl esters, no inhibition of $\beta\beta$ occurs at concentrations that cause inhibition of up to 80% of other Class I isozymes. This difference in response to ester inhibitors is mirrored by kinetic differences seen between $\beta\beta$ and other Class

I isozymes. For both alcohols and aldehydes, k_{cat} and K_m values for the ββ isozyme deviate from those measured for other isozymes of its class.

ADH inhibition substantially effects ethanol metabolism of animals (20,21,24,25), and suitable inhibitors could be valuable for the study of ethanol metabolism and preventive therapy of methanol and ethylene glycol poisoning (26). Pyrazoles, in particular 4-methylpyrazole, are potent inhibitors and thereby decrease the rate of ethanol elimination in ethanol intoxicated rats (24,25). Amides also inhibit ADH; isobutyramide, other N-alkylformamides, p-butoxyphenyl acetamide and N-(p-butoxybenzyl)formamide have been studied as inhibitors both in vitro and in vivo (20,21). In principle, therefore, inhibitors would not be expected to off-set intoxication, but to prolong it by extending the lifetime of ethanol.

In hepatocyte preparations from ethanol-intoxicated rodents, the presence of Narcan® was found to restore the intracellular NAD^+/NADH ratio to normal, which-during intoxication-is decreased by higher concentrations of the reduced nucleotide (27,28). From this observation it was argued that if the availability of NAD^+ were rate-limiting, then the increase in NAD^+ concentration caused by naloxone would facilitate ethanol metabolism, leading to rapid clearance of ethanol from the body (27). Alternatively, the effect of Narcan® on NAD^+ concentrations in hepatic tissue could result from inhibition of alcohol dehydrogenase (ADH), the first enzyme in the metabolism of ethanol. If the reduction of NAD^+ to NADH during ethanol oxidation by ADH is halted, the ratio of NAD^+/NADH is expected to return to normal; this is the effect observed for Narcan®.

Narcan® alters the effects of ethanol metabolism of impaired cells and, perhaps, of animals. The evidence presented here shows that Narcan® contains esters that inhibit all classes of ADH isozymes. Hence, the response of cells or animals to Narcan® in conjunction with ethanol intoxication can neither be attributed to the role of naloxone nor to the enhancement of ADH activity. In hepatocytes, methylparaben at concentrations above its K_I for ADH can account fully for the action of Narcan® (29).

Results of studies on the efficacy of Narcan® in reversing ethanol intoxication in humans and rodents are conflicting. Such data and their interpretation deserve reevaluation since possible antagonistic effects of naloxone on the consequences of ethanol oxidation may be obscured by the inhibition of ADH, which would potentiate intoxication.

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