Biochemical Pharmacology, Vol. 57, pp. 511–520, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00325-6

The High-Affinity Binding of [^{3}H]Norharman ([^{3}H]β-carboline) to the Ethanol-Inducible Cytochrome P450 2E1 in Rat Liver

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ABSTRACT. High-affinity binding sites of [^{3}H]norharman (synonymous: [^{3}H]β-carboline) were characterized in microsomal membranes from rat liver utilizing various β-carboline (BC) derivatives and substances binding to enzymes of the cytochrome P450 (*CYP*) superfamily (EC 1.14.14.1). Saturation experiments demonstrated that [^{3}H]norharman binds with high-affinity (dissociation constant 20.86 nM; maximum binding 21.40 pmol/mg protein). Displacement experiments with the β-carboline derivatives 6-methyl-BC and 6-hydroxy-BC revealed a better adaptation to the two-site model, indicating that [^{3}H]norharman binds to at least two sites, with an affinity of the high-affinity site in the low nM range. Substances binding with relative preference to isozymes of the *CYP* superfamily displaced [^{3}H]norharman with a lesser potency than unlabeled norharman. Imidazole, pyrazole, and 4-methylpyrazole, known as inducers of the ethanol-inducible *CYP*2E1, displaced [^{3}H]norharman with relative high potency. Furthermore, binding experiments with microsomes from human lymphoblast-expressed rat *CYP*2E1 revealed a high-affinity binding site [inhibition constant (K_i) 13.21 nM] comparable to that of microsomal membranes for norharman. It was displaceable by ethanol (K_i 14.25 μM), indicating that norharman and ethanol bind to the same binding site on *CYP*2E1. *In vivo* experiments with rats which had ingested ethanol for two weeks revealed that norharman blood plasma levels were significantly elevated at the end of this period, supporting the notion of an interaction of norharman and ethanol metabolism. Since it has been demonstrated in the Ames test that norharman's comutagenic action is connected with microsomal membranes (containing *CYP* isozymes), the present findings suggest that the observed increase in the levels of norharman in alcoholics leads to further *CYP* enzyme induction and thereby contributes to the increased risk of carcinomas in these patients. BIOCHEM PHARMACOL **57**;5:511–520, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. [^{3}H]norharman; β-carboline; rat liver; cytochrome P450 2E1; ethanol; cancer

Several BCs† have been identified in plants and foodstuff, and as natural compounds in mammals [1–5]. The biosynthesis of the 1-methyl-BCs occurs via a condensation reaction of pyruvate and indoleamines in rats [6, 7], whereas the BCs lacking the methyl group derive from indoleamines and formaldehyde [8]. BCs have also been isolated in tobacco smoke (80 nmol/g tobacco; [9]) and fried meat [10] as substances formed from tryptophan after protein pyrolysis. Harman (1-methyl-β-carboline) has been identified as a potent endogenous MAO-A inhibitor, thereby possibly exerting antidepressant properties [11, 12]. Norharman (synonymous: β-carboline) is known to inhibit MAO-B with a relatively low potency [13]. Moreover, it was recently shown that harman and norharman activate GTP binding proteins in a receptor-independent manner [14]. Norharman is found in the human brain with its highest concentration in the substantia nigra (16 ± 8 pmol/g tissue). *N*-methylated ("bioactivated") BCs have been implicated in the pathogenesis of Parkinson's disease as a possible endogenous factor [15]. High-affinity [^{3}H]norharman binding sites have been demonstrated in rat brain as being neither identical with the benzodiazepine receptor nor MAO [16]. Harman displaces high-affinity [^{3}H]norharman binding in rat brain (K_i 1.7 nM; [16]). However, harman, MAO inhibitors, benzodiazepine receptor ligands, and compounds known to bind to the D_2 and "sigma" receptors do not bind to the high-affinity [^{3}H]norharman binding sites in liver tissue [17].

Besides their potential role in the pathogenesis of Parkinson's disease, BCs have also been demonstrated to be elevated in alcoholic patients, even after a period of abstinence, and have therefore been postulated as state and residual markers of alcoholism [18]. Abuse of alcoholic beverages is associated with a higher incidence of cancers

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† *Abbreviations:* BC, β-carboline (synonymous: norharman); PNC, 5-pregnen-3β-O1-20-one-16α-carbinitrile; 6-MeO-THN, 6-methoxy-tetrahydronorharman; *CYP*, cytochrome P450; K-MOPS, sodium-3-*N*-morpholino-propansulfonic acid; MAO, monoamine oxidase; K_d, dissociation constant; K_i, inhibition constant; and B_{max}, maximum binding.

Received 24 February 1998; accepted 2 August 1998.

[19], especially of the gastrointestinal tract. Besides other mechanisms, the bioactivation of procarcinogens by *CYP* isozymes (EC 1.14.14.1) plays an important role in the pathogenesis of alcohol-related cancer [20]. Norharman is known to be comutagenic, but is not a mutagen itself [21]. Therefore, its bioactivation of *CYP* isozymes might contribute to the higher incidence of cancer in alcoholic patients. The aim of the present study was to further characterize the [^{3}H]norharman binding sites in rat liver membranes with respect to their possible interference with bioactivating enzymes, namely the *CYP* isozymes, as well as with the metabolism of ethanol. The possible consequences for the pathogenesis of cancer will be discussed.

MATERIALS AND METHODS

Animals

Liver from male Wistar rats 6–10 months old (Lippische Versuchstierzucht) was used for binding experiments. Three to four animals were housed in macrolon cages in an air-conditioned animal room (temperature 21°; 50% humidity; 12hr/12hr-light/dark cycle). The rats received a standard diet (Altromin 1324) and tap water *ad lib*.

Wistar rats 6–10 months old (Lippische Versuchstierzucht) used for forced ethanol ingestion were housed individually in macrolon cages (43×26×15 cm) in an air-conditioned animal room (temperature 21°; 50% humidity; 12hr/12hr-light/dark cycle). They were fed a diet (Altromin C 0200, highly digestible, enriched in vitamins, trace elements, and amino acids) dissolved in 6.5% (v/v) ethanol for 28 days (N = 20). The respective control rats (N = 10) were housed under the same conditions and received the diet dissolved in water. The weight of the animals and the amount of ethanol (forced rats) and water (controls), respectively, consumed were recorded three times a week. On day 28, 6 of the ethanol liquid diet rats were decapitated and the blood collected. The other rats received water instead of the ethanol liquid diet. A second group was decapitated at day 8 (N = 6) and the third group at day 28 (N = 8) postwithdrawal. Control rats (N = 10) were decapitated at day 28 of the postwithdrawal period. To assess whether the Altromin diet contains harman and norharman, we dissolved an aliquot of the diet in water, ran it through the assay (see below), and did not detect either of the BCs.

Subcellular Fractionation of Rat Liver Membranes

Rat liver was dissected and homogenized in 10 volumes (w/v) of ice-cold 0.32 M sucrose using a glass homogenizer with a Teflon pestle (Braun-Melsungen). The homogenate was centrifuged at 1000 *g* and 4° for 10 min (Sorvall RC5C, DuPont). Subsequently, the pellet was resuspended in sucrose and centrifuged as described above, resulting in the nuclear fraction (P1 pellet), which was discarded. The supernatants from both centrifugation steps were combined and centrifuged at 23,000 *g* and 4° for 30 min (P2 pellet). The resulting supernatant was centrifuged at 100,000 *g* and 4° for 60 min (Beckman L2-65B), resulting in the crude microsomal fraction (P3 pellet) and the supernatant containing the cytosol. The P3 pellet was resuspended in 25 mM K-MOPS buffer (pH 7.4), homogenized, and then stored at −80° in plastic tubes until further use.

Binding Assay

The binding assay with [^{3}H]norharman and crude microsomal (P3) membranes of rat liver was performed according to a method previously described by May *et al.* [22]. All incubations were conduced on ice (0°). Nonspecific binding of [^{3}H]norharman was defined by coincubation with 10 μM unlabeled norharman, a concentration approximately 100-fold higher than the K_i value determined in displacement studies (see below). The incubation mixture contained: 100 μL of 50 mM K-MOPS incubation buffer (pH 7.4), 100 μL H_2O (defining total binding); 100 μL of 50 μM norharman respectively (defining nonspecific binding); and 200 μL crude (P3) microsomal membranes of rat liver and 100 μL [^{3}H]norharman (dissolved in H_2O; mean concentration ~4 nM), starting the incubation. The amount of protein used in binding assays was ~20 μg. The incubation lasted 90 min and was terminated by filtration under vacuum using a Brandel cell harvester (Brandel Inc.) to collect the membranes on Whatman GF/B filters (Dunn Labortechnik) presoaked in 0.1% polyethyleneimine for 1 hr to minimize nonspecific binding. Filtration was followed by three washings with 5 mL ice-cold 50 mM Tris–HCl buffer (pH 7.4). Radioactivity remaining on the filters was measured by liquid scintillation spectrometry with an efficiency of about 55% (Packard 1900 CA).

Saturation experiments were conducted with 15–18 different concentrations of [^{3}H]norharman (range 0.2–91.5 nM). Increasing concentrations of various pharmacological substances were tested in displacement studies. Nonspecific binding was again defined by coincubation of 10 μM unlabeled norharman.

[^{3}H]Norharman displacement studies using microsomes from human lymphoblast-expressed rat *CYP*2E1 and controls were performed in the same way as described above. The amount of the protein in the incubation mixture was 20 μg.

Determination of Norharman and Harman in Blood Plasma

Blood plasma samples (~6–9 mL blood) for the measurement of norharman and harman were collected in plastic tubes containing 2000 IE LIQUEMIN® (Hoffmann La-Roche). The samples were centrifuged at 300 *g* and 10° for 15 min without brake (Minifuge Heraeus). The supernatants (platelet-rich plasma) were carefully removed and centrifuged at 27,000 *g* and 4° for 10 min. The resulting supernatants (plasma) were decanted and stored at −80°.

After thawing, plasma samples were centrifuged at

33,000 *g* and 4° for 10 min (Sorvall, RC5C). Norharman and harman blood plasma levels were determined using HPLC with fluorescence detection as described earlier [23].

Protein Assay

Protein concentrations of crude microsomal (P3) membrane fractions from rat liver were determined using the bicinchoninic acid protein assay kit purchased from Sigma Chemie.

Materials

[6-^{3}H]Norharman (specific activity 24–50 Ci/mmol) was custom-labeled by Amersham International, England, by halogen–tritium exchange with 6-Br-norharman (gift from Dr. Heineke, University of Bonn, Germany). The following drugs were purchased from Sigma Chemie: harman, norharman, indole-3-carbinol, 20-methylcholanthrene, trans-stilbene-oxide, 5′5-diphenylhydantoin, PNC, isoniazide, pyrazole, 4-methylpyrazole, imidazole, dexamethasone, spironolactone, erythromycin, rifampicin, coumarine, chlorpromazine, bicinchoninic acid solution and copper (II) sulfate, BSA, MOPS, Tris, and polyethyleneimine. Quinidine was purchased from RBI Bio Trend Chemicals and diethyldithiocarbamate was from Aldrich Chemicals. Sucrose and DMSO were purchased from Merck and ULTIMA GOLD® scintillation fluid from Packard. 6-Methoxy-THN was synthesized by Ms. S. Strauβ (Dept. of Clinical Neurobiology, Free University of Berlin, Germany). 6-Methyl-norharman and 6-hydroxy-norharman were gifts from Prof. J. Lehmann (University of Bonn, Germany) and 2-methyl-norharman was a gift from Prof. M. A. Collins (Loyola University, IL). All other chemicals were purchased from readily available commercial sources and were of the highest purity grade available.

Human lymphoblast-expressed rat CYP2E1 microsomes (*p*-nitrophenol hydroxylase activity 420 pmol/min × mg protein) and control lymphoblasts (expressing no CYP2E1 activity) were purchased from GENTEST Corp. (NatuTec).

The following substances were dissolved in DMSO: 20-methylcholanthrene, 6-methyl-BC, and 6-hydroxy-BC (in 2%) and trans-stilbene-oxide, dexamethasone, spironolactone, erythromycin, and rifampicin in 1%. Coumarine was dissolved in 1% in displacement studies, and PNC and coumarine in 0.5% in screening experiments. The respective controls were treated in the same manner with DMSO.

Data Analysis

Equilibrium binding parameters (K_d and B_{max}) and inhibition constants (K_i) were calculated by nonlinear regression analysis of the nontransformed data using the GraphPad Prism program purchased from GraphPad Software. Statistical significance of the two-site binding model was established if the P value was <0.05. R^2 was 0.71–0.97 in saturation plots and at least 0.98 in displacement experiments. Hill coefficients (n_H) where determined by linear regression analysis of Hill plots. Data from animal experiments were analyzed using the paired t-test.

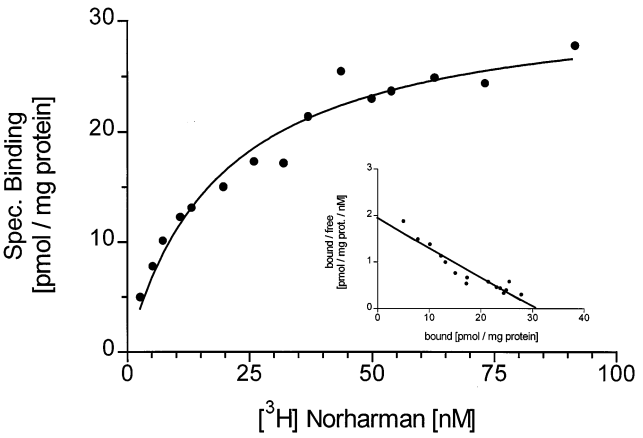


FIG. 1. Saturation kinetics of high-affinity [^{3}H]norharman binding in microsomal membranes of rat liver. Incubation of increasing concentrations of [^{3}H]norharman resulted in a hyperbolic curve for specific binding. Findings of a representative experiment are shown. The inset demonstrates the same data after Scatchard transformation. Corresponding binding parameters were: K_d 20.86 ± 2.55 nM; B_{max} 21.40 ± 6.01 pmol/mg protein; n_H 0.97 ± 0.07 (N = 5).

RESULTS

Kinetics of [^{3}H]Norharman Binding

Incubation of crude microsomal (P3) membrane fractions from rat liver with 15–18 different concentrations of [^{3}H]norharman (minimum 0.2 nM–maximum 91.5 nM) demonstrated a limited number of binding sites (B_{max} 21.40 ± 6.01 pmol/mg protein, mean ± SEM; N = 5). A hyperbolic shape of the saturation curve was obtained for specific binding of [^{3}H]norharman (Fig. 1). There appears to be a homogenous binding site for [^{3}H]norharman at microsomal membranes of rat liver, indicated by linearity after Scatchard transformation [24] and corresponding Hill coefficients being near unity (n_H 0.97 ± 0.07, mean ± SEM; N = 5). Nonlinear regression analysis of [^{3}H]norharman saturation plots yielded a single high-affinity binding site, with a K_d of 20.86 ± 2.55 nM (mean ± SEM; N = 5).

Displacement Studies with β-Carbolines

Norharman displaced [^{3}H]norharman with high-affinity from its binding site at microsomal (P3) membranes (Fig. 2A, Table 1). Analysis of norharman displacement experiments resulted in monophasic inhibition curves ($K_i \sim 35$ nM) with Hill coefficients near unity. Harman was not able at all to displace [^{3}H]norharman from its binding site. According to their potency, the BCs could be ranked as follows: 6-methyl-BC > 6-hydroxy-BC >/= norharman > 2-methyl-BC > 6-MeO-THN, with harman being inactive. 2-Methyl-BC and 6-MeO-THN inhibition curves were

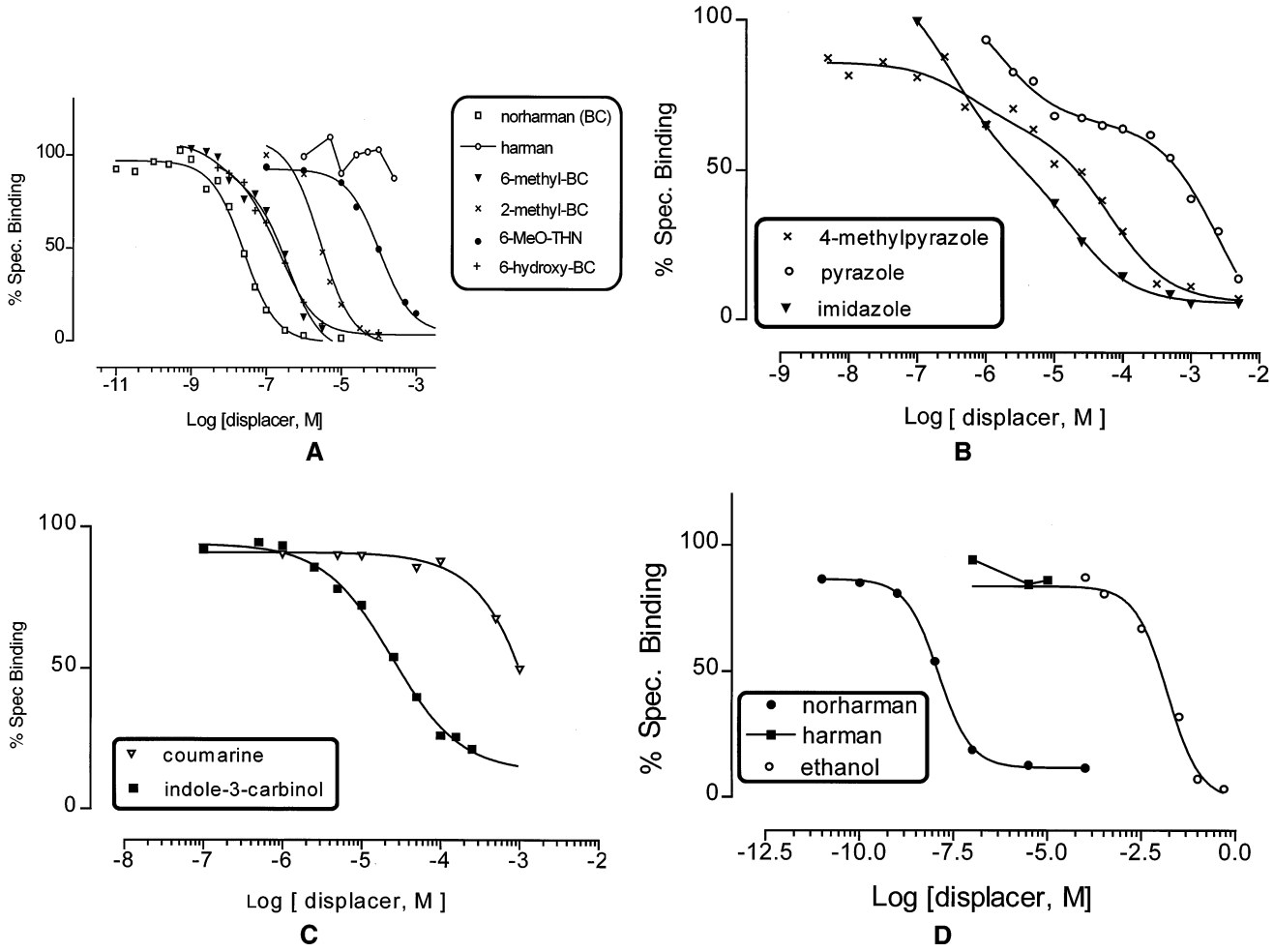


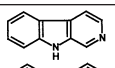
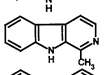
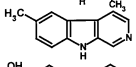
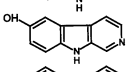
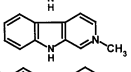
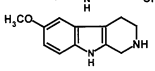
FIG. 2. (A) Representative displacement curves of specific [^{3}H]norharman binding to microsomal membranes of rat liver by different β-carboline derivatives are depicted. The [^{3}H]norharman concentration was approximately 4 nM; nonspecific binding was defined by the presence of 10 μM norharman. The corresponding values of the inhibition constants (K_i) are compiled in Table 1. (B) Representative displacement curves of specific [^{3}H]norharman binding by different CYP2E1 ligands to microsomal membranes are depicted. The [^{3}H]norharman concentration was approximately 4 nM; nonspecific binding was defined by the presence of 10 μM norharman. The corresponding values of the inhibition constants (K_i) are compiled in Table 3. (C) Representative displacement curves of specific [^{3}H]norharman binding by indole-3-carbinol (CYP1A1/2) and coumarine (CYP2A6) to microsomal membranes are depicted. Displacement studies with coumarine were limited by its poor solubility. The [^{3}H]norharman concentration was approximately 4 nM; nonspecific binding was defined by the presence of 10 μM norharman. The corresponding values of the inhibition constants (K_i) are compiled in Table 3. (D) Representative displacement curves of specific [^{3}H]norharman binding by norharman, harman, and ethanol to human lymphoblast-expressed rat CYP2E1 microsomes are depicted. The [^{3}H]norharman concentration was approximately 4 nM; nonspecific binding was defined by the presence of 10 μM norharman. The K_i value for norharman was 15.96 nM, n_H 0.85 (N = 2). The K_i value for ethanol was 15.35 μM, n_H 0.61 (N = 2). Harman showed no significant [^{3}H]norharman displacement.

monophasic (Hill coefficients ~1), with 2-methyl-BC (K_i ~ 4 μM) being an approximately 120-fold weaker displacer than norharman. Next to it in potency was 6-MeO-THN (K_i ~ 70 μM). Two exceptions to monophasic displacement curves were 6-methyl-BC and 6-hydroxy-BC. Computer analysis of the displacement curves of these substituted BCs with short substituents at position 6 revealed a better adaptation to a two-site model, with Hill coefficients significantly smaller than unity, indicating a more complex interaction with the [^{3}H]norharman binding site. A high-affinity K_i-1 value (K_i-1 ~ 3 nM) was calculated for 6-methyl-BC, being about 10-fold smaller than the respective value of norharman. The corresponding low-affinity K_i-2 value was ~ 500 nM, with 60% contribution of the low-affinity site. 6-hydroxy-BC (K_i-1 ~ 24 nM) was as potent as norharman, but 8-fold weaker than 6-methyl-BC. The corresponding low-affinity K_i-2 value was ~ 300 nM. About 40% contributed to the high-affinity binding site of 6-hydroxy-BC.

Displacement Studies with CYP Ligands

Before performing detailed displacement experiments, we examined various ligands with known preference for specific *CYP* isozymes in a screening manner for further selection. Only ligands known to bind to xenobiotica-

TABLE 1. Displacement of specific [^{3}H]norharman binding from microsomal (P3) membranes of rat liver by different β-carboline derivatives

Ligand		K_i-1 (nM) (±*SEM*)	% binding sites K_i-1 (±*SEM*)	K_i-2 (nM) (±*SEM*)	% binding sites K_i-2 (±*SEM*)	n_H (±*SEM*)	n
Norharman		35.55 ±6.31	-	-	-	1.06 ±0.01	4
Harman		-	-	-	-	-	5
6-Methyl-BC		3.22	39	508.55	61	0.38	2
6-Hydroxy-BC		24.25 ±14.21	44.75 ±7.95	345.15 ±98.07	55.25 ±7.95	0.77 ±0.07	4
2-Methyl-BC		4200	-	-	-	1.02	2
6-MeO-THN		76000	-	-	-	0.84	2

Shown are the results [inhibition constant (K_i), Hill coefficients (n_H), and the percentage of binding sites] of [^{3}H]norharman displacement experiments using different β-carbolines and microsomal membranes from rat liver. Data are the means ±SEM.

metabolizing *CYP* isozymes were included. Several of the tested compounds displaced [^{3}H]norharman from its binding sites at microsomal (P3) membranes with some potency. Most of the compounds were difficult to dissolve in water, causing limitations with respect to the testing of high concentrations. Ligands of the *CYP*1A1/2, *CYP*2A6, *CYP*2D6, and *CYP*2E1 families exerted the most potent inhibition (Table 2).

Analysis of inhibitors of *CYP*3A1 was limited by their poor solubility in water, but erythromycin, a "prototype" ligand for *CYP*3A1, showed no effect on [^{3}H]norharman binding at a concentration of 100 μM. Therefore, erythromycin was not further investigated.

Selected by screening tests, several ligands, known to bind relatively specifically to *CYP* isozymes, were also found to be potent inhibitors of specific [^{3}H]norharman binding in displacement studies (Table 3). Computer analysis of displacement experiments with *CYP*2E1 ligands showed a better adaptation to the two-site binding model. Inhibition curves with the *CYP*2E1 inhibitors imidazole, pyrazole, and its derivative 4-methylpyrazole were biphasic, with Hill coefficients significantly lower than unity (Fig. 2B).

Imidazole was the strongest displacer of [^{3}H]norharman, with an apparent high-affinity K_i-1 value of 0.24 μM (~45% of the total number of specific binding sites) and a low-affinity K_i-2 value of 12.73 μM. The high-affinity K_i-1 value of 4-methylpyrazole was similar to that of imidazole (0.27 μM) and the low-affinity site was slightly less potent (K_i-2 19.95 μM). Pyrazole was a much weaker displacer of [^{3}H]norharman binding than its derivative 4-methylpyrazole, having an approximate 8-fold weaker affinity (K_i-1 2.2 μM) to the high-affinity binding site and a 60-fold lower affinity (K_i-2 ~ 1.2 mM) to the low-affinity binding site. For both pyrazole and 4-methylpyrazole, the distribution of high- and low-affinity binding sites was the same, with ~30% of the binding sites having high-affinity. Indole-3-carbinol, a *CYP*1A1/2 inhibitor, was the only tested *CYP* ligand with a monophasic inhibition curve and corresponding Hill coefficients near unity (Fig. 2C). Its K_i value (17.94 μM) was 8-fold larger than the high-affinity K_i-1 of the pyrazole inhibition. Displacement studies with coumarine, a *CYP*2A6 ligand, were limited by its poor solubility in incubation buffer. At the concentrations tested, coumarine

TABLE 2. Displacement of specific [^{3}H]norharman binding from microsomal (P3) membranes of rat liver by various compounds known to bind with preference to certain cytochrome P450 isozymes

Ligand	Final concentration	% inhibition of spec. binding	% range of inhibition	n
CYP1A1/2				
Indole-3-carbinol	100 μM	60.1	59.8–60.4	2
20-Methylcholanthrene	2 μM	13.6	13.2–14.0	2
CYP2A6				
Coumarine	100 μM	33.2	29.7–36.7	2
CYP2B1/2				
5′5-Diphenylhydantoin	100 μM	30.5	29.6–31.4	2
Trans-stilbene-oxide	15 μM	38.7	34.3–43.2	2
CYP2D6				
Quinidine	100 μM	29.9	29.0–30.8	2
Chlorpromazine	100 μM	46.6	45.2–48.0	2
CYP2E1				
Isoniazide	100 μM	33.6	24.3–43.0	2
Pyrazole	100 μM	49.6	44.6–54.7	2
4-Methylpyrazole	100 μM	65.1	53.7–76.5	2
Imidazole	100 μM	86.1	85.9–86.3	2
Diethyldithiocarbamate	100 μM	0.0	0.0	2
CYP3A1				
Dexamethasone	20 μM	24.9	14.3–35.6	2
PNC	3 μM	14.7	14.5–15.0	2
Spironolactone	11 μM	5.0	0.0–10.0	2
Erythromycin	100 μM	4.6	0.0–9.2	2
Rifampicin	10 μM	10.5	7.0–14.1	2

Compiled are the results of displacement screening experiments using ligands of cytochrome P450 isozymes with [^{3}H]norharman and microsomal membranes from rat liver. Data are the means of the percentage of inhibition of two separate experiments. The final concentration of the displacing compound used in the experiments is indicated.

TABLE 3. Displacement of specific [^{3}H]norharman binding from microsomal (P3) membranes of rat liver by cytochrome P450 ligands

Cytochrome P450 ligands	K_i-1(μM)	% binding sites K_i-1	K_i-2(μM)	% binding sites K_i-2	n_H	n
Imidazole	0.24	44.5	12.73	55.5	0.60	2
4-Methyl-pyrazole	0.27	24.5	19.95	75.5	0.53	2
Pyrazole	2.20	35	12000	65	0.35	2
Indole-3-carbinol	17.94	-	-	-	1.26	2
Coumarine	~1900	-	-	-	-	2

Results of [^{3}H]norharman displacement studies using microsomal membranes from rat liver with the cytochrome P450 ligands imidazole, pyrazole, and 4-methylpyrazole (*CYP*2E1), indole-3-carbinol (*CYP*1A1/2), and coumarine (*CYP*2A6). Also presented are the percentage of binding sites having high- and low-affinity, respectively, for the ligand. Data are the mean values of two individual experiments. Experiments with coumarine were limited by its poor solubility, so K_i values and Hill coefficients could only be calculated approximately. Hill coefficients are designated (n_H).

reduced specific [^{3}H]norharman binding by only 50% (Fig. 2C). Therefore, inhibition constants (K_i) could only be calculated approximately. Other compounds which had affinity to the [^{3}H]norharman binding site in screening experiments (Table 2), such as the *CYP*2B1/2 ligand trans-stilbene-oxide and the *CYP*2D6 ligands quinidine and chlorpromazine, did not significantly affect specific [^{3}H]norharman binding in displacement studies.

[^{3}H]Norharman Displacement by Norharman, Harman, and Ethanol at CYP2E1 *from Human Lymphoblasts*

Displacement studies with [^{3}H]norharman using microsomes from human lymphoblast-expressed rat *CYP*2E1 revealed a high-affinity binding site for norharman, with a K_i value of 13.21 nM and a Hill coefficient (n_H) of 0.97 (N = 2; Fig. 2D).

No specific binding of [^{3}H]norharman to *CYP*2E1 was detected after preincubation of the lymphoblasts for 15 min at 100° (N = 2) and with control lymphoblasts not expressing *CYP*2E1 (N = 2). Ethanol demonstrated [^{3}H]norharman displacement in a concentration-dependent manner, but with much less affinity than norharman (K_i 14.25 μM; n_H 0.74; N = 2). Computer analysis revealed a better adaptation to the one-site model for norharman and ethanol displacement. Harman did not significantly displace [^{3}H]norharman from its binding site on *CYP*2E1 (N = 2).

In Vivo *Studies*

To assess whether chronic application of ethanol affects the blood levels of norharman and specifically whether the induction of *CYP*2E1 by ethanol interacts with the metabolism of norharman, male Wistar rats were forced to drink ethanolic solutions for 28 days. The rats ingested 4.9 g of ethanol daily on average and a total amount of fluid of 27 ± 0.4 mL, with their body weight increasing from 270 ± 2.4 g to 326 ± 2.6 g. On the last day of ethanol ingestion (9 a.m.), the levels of norharman were elevated (30.9 ± 5.1 pg/mL blood plasma, mean ± SEM, N = 6) compared to control rats (15.4 ± 3.9 pg/mL blood plasma, mean ± SEM, N = 10), whereas at day 28 postwithdrawal the norharman levels were significantly reduced (5.1 ± 3.36 pg/mL blood plasma, mean ± SEM, N = 8; Fig. 3).

The respective values for the rats at day 8 of withdrawal were: 26.0 ± 9.2 pg/mL blood plasma, mean ± SEM, N = 6. No significant changes in the harman levels were found. On the last day of ethanol ingestion (9 a.m.), harman was not elevated (9.02 ± 3.5 pg/mL blood plasma, mean ± SEM, N = 6) compared to controls (8.56 ± 3.71 pg/mL blood plasma, mean ± SEM, N = 10). No statistically significant change in harman levels was found at day 8 postwithdrawal (4.6 ± 2.6 pg/mL blood plasma, mean ± SEM, N = 6) and at day 28 postwithdrawal (5.7 ± 2.35 pg/mL blood plasma, mean ± SEM, N = 8).

DISCUSSION

Norharman (BC) has been demonstrated to be elevated in the blood plasma and urine of alcohol-dependent patients [23, 25]. Furthermore, it is well recognized that alcohol

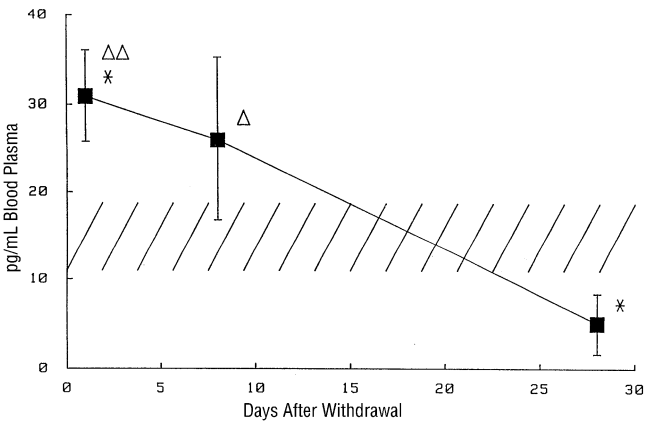


FIG. 3. Effects of a forced ethanol liquid diet (4.9 g daily on average for 28 days) on the norharman (BC) blood plasma levels of Wistar rats on the last day of ingestion (day 1; 9 a.m.), day 8, and day 28 following postwithdrawal. The cross-hatched area denotes the range ±SEM of controls exposed to liquid diet without ethanol. On the last day of ethanol ingestion, norharman levels were significantly elevated, but on day 28 postwithdrawal, they were significantly lower compared to controls. The norharman levels at day 1 and 8 were higher than those at day 28. *$P < 0.05$ compared with controls; $^{\Delta\Delta}P < 0.007$; and $^{\Delta}P < 0.05$ compared with levels at day 28.

abuse poses a high risk of cancer [19]. This association might be of importance, because norharman has been found to be comutagenic, while not being mutagenic itself [21]. One proposed mechanism of the comutagenic action of β-carbolines is their direct interaction with DNA. Harman (1-methyl-BC) has been found to intercalate into DNA more easily than norharman [26], but the latter is the more potent comutagen according to the Ames test [27]. Thus, intercalation into DNA does not fully explain the comutagenic action of BCs. Various compounds have been found whose biological activation to ultimative mutagens by norharman requires the presence of microsomal enzymes (e.g. *CYP* isozymes) in the Ames test. The *CYP* isozymes metabolize a wide range of endogenous compounds and xenobiotica, including alkaloids [28]. Today, more than 400 different *CYP* isozymes have been identified [29] which can be induced by various compounds [30]. *CYP*s are known as the key enzymes in the metabolic activation of chemical carcinogens and toxins [31]. As was previously reported, norharman interacts with the steroidogenic cytochromes *CYP*11 in rat adrenal mitochondria and *CYP*17 in rat testicular microsomes. Progesterone binding to *CYP*17 was competitively inhibited by norharman (K_i 2.6 μM), whereas harman, tetrahydroharman, and tetrahydronorharman had no effect up to a concentration of 200 μM [32]. Moreover, our laboratory reported high-affinity binding sites of [^{3}H]norharman at rat liver membranes, with its highest density in the microsomal membrane fraction [22]. Therefore, binding of norharman to *CYP* enzymes is not only interesting with regard to BC metabolism, but could also partly explain its comutagenic action.

Analysis of [^{3}H]norharman saturation plots revealed a high-affinity, single binding site at microsomal membranes from rat liver, with K_d values in the low nM range. As is indicated by linear Scatchard plots with corresponding Hill coefficients near unity, [^{3}H]norharman seems to bind to a homogenous population of binding sites. [^{3}H]norharman was displaced by addition of unlabeled norharman and several 2- and 6-substituted BC derivatives. As reported in a previous study [22], we could confirm that harman does not affect high-affinity [^{3}H]norharman binding sites in rat liver, which supports the notion of different high-affinity [^{3}H]norharman binding sites in brain and liver tissue. This is remarkable, because harman differs from norharman only by a methyl substituent on the C1-position. Harman, a potent MAO-A inhibitor, is known to bind to rat liver, but its highest density of binding sites is located at mitochondrial membranes [22]. Methyl- or hydroxy- substitution of BCs at positions 2 or 6 did not prevent affinity to the [^{3}H]norharman binding sites at microsomal membranes of rat liver. Analysis of the [^{3}H]norharman inhibition by 6-methyl-BC and 6-hydroxy-BC revealed displacement with best fit to the two-site model. The inhibition curves were biphasic, with Hill coefficients smaller than unity.

The K_i-1 value of 6-methyl-BC and 6-hydroxy-BC indicated an even higher or equal affinity than that of norharman itself. High- and low-affinity binding sites were nearly equally distributed. This result seemed to be in contrast to the linear Scatchard plots of [^{3}H]norharman binding, because Hill coefficients smaller than unity indicate a more complex interaction between ligand and binding sites, which may result from a heterogenous population of binding sites, cooperativity, or a two-step/three-component binding system [33]. However, selectivity is only detected if the two classes of binding sites differ in their affinity for the ligand by at least 5–10 fold [33].

Because ligands for the steroidogenic and cholesterol-metabolizing *CYP*s, such as testosterone and estradiol, share some structural features with BCs, we investigated these ligands and found that they were inactive under the conditions of our assay.

Therefore, we directed our attention to the inducible *CYP* isozymes, which can be classified according to Okey [30] by their preferred ligand into the "polycyclic aromatic compound type" (*CYP*1A1/2), the "phenobarbital type" (*CYP*2B1/2B2), the "glucocorticoid type" (*CYP*3A1), and the "ethanol type" (*CYP*2E1). In our screening tests, several ligands from these categories were found to inhibit [^{3}H]norharman binding. Experiments with ligands of the *CYP*3A1 enzyme family, namely dexamethasone, PNC, spironolactone, and rifampicin, were limited by poor solubility of the ligands in water, but erythromycin, a "prototype" ligand of *CYP*3A1 [30], did not affect [^{3}H]norharman binding to microsomal rat liver membranes at a concentration of 100 μM. Therefore, these ligands were not further investigated. Apart from indole-3-carbinol, an inducer of *CYP*1A1/2 [30], moderate to high affinity to [^{3}H]norharman binding sites was found by different inhibitors of the *CYP*2 subfamilies and their individual forms (*CYP*2A6, *CYP*2D6, and *CYP*2E1) in screening tests. We explain the affinity of different inhibitors by the high overlap in amino acid structure between cytochrome families and subfamilies [28].

To further characterize the interaction of these ligands with the [^{3}H]norharman binding sites, we used different ligands after selection by screening tests. Among these, the ligands known to induce *CYP*2E1 severalfold, such as imidazole [34], 4-methylpyrazole, and pyrazole [35], demonstrated high affinity, indicative of [^{3}H]norharman binding to *CYP*2E1. 4-Methylpyrazole was more potent in displacing [^{3}H]norharman than pyrazole, which is in accordance with the results of others, who demonstrated a more potent *CYP*2E1 induction by 4-methylpyrazole [36] and the stronger inhibition of microsomal ethanol oxidation by 4-methylpyrazole (K_i 0.7 mM) than pyrazole (K_i 1.1 mM) [37]. Like the 6-substituted BCs, all *CYP*2E1 ligands displaced [^{3}H]norharman in a biphasic manner, with Hill coefficients smaller than unity. The other *CYP*2E1 ligands investigated in screening experiments were diethyldithiocarbamate and isoniazide. Diethyldithiocarbamate is a known "mechanism-based inhibitor" of *CYP*2E1 [38], which explains its missing action in our *in vitro* experiments.

*CYP*2E1 contributes the major bulk to the microsomal ethanol oxidizing system, the main nonalcohol dehydroge-

nase pathway of ethanol metabolism. It also catalyzes the metabolism and biological activation of many low molecular weight compounds commonly found as solvents at home and in industry or in tobacco smoke [38]. *CYP*2E1 has been identified as the major microsomal *N*-nitrosodimethylamine demethylase [39] and norharman is comutagen with different *N*-nitrosamine derivatives [40]. Thus, interaction of norharman with the ethanol-metabolizing *CYP*2E1, which also takes part in the biological activation of mutagens and carcinogens, might be of major clinical importance in light of the known elevated BC levels in subgroups of alcoholic patients and the well-known higher incidence of cancer in these patients.

To further investigate norharman's interaction with the ethanol-inducible *CYP*2E1, we conducted binding experiments with human lymphoblast-expressed rat *CYP*2E1 microsomes. This cell line, which expresses a single *CYP* cDNA, offers the potential to analyze *CYP* form-specific binding, avoiding the issue of *CYP* purity and the possibility of antibody cross-reacting with other *CYP* isoforms [41]. Furthermore, an appropriate control cell line is readily available. Binding experiments with lymphoblast-expressed *CYP*2E1 revealed a single, high-affinity binding site for norharman with a K_i in the low nM concentration range, one comparable to the K_i values of norharman at microsomal membranes of rat liver. Furthermore, [^{3}H]norharman binding to *CYP*2E1 was displaceable by ethanol in a concentration-dependent manner, indicating that norharman and ethanol bind to the same site on the enzyme. Still, the affinity of ethanol was much lower than that of norharman, but it is know that ethanol has a rather low affinity to *CYP*2E1. No specific [^{3}H]norharman binding was detected with control lympoblasts and after pretreatment of human lymphoblast-expressed *CYP*2E1 microsomes at 100° for 15 min. The high-affinity K_d and K_i values of norharman are remarkable, but when comparing the results of the enzyme tests, it has to be kept in mind that they are performed at different temperatures (0° and 37°, respectively). Higher incubation temperatures might decrease the affinity of ligands.

Besides the *CYP*2E1 ligands, indole-3-carbinol, which induces *CYP*1A1/2 mRNA synthesis in rat liver [42], inhibited [^{3}H]norharman binding at microsomal membranes of rat liver with monophasic inhibition curves, but to a lesser extent than imidazole, pyrazole, and 4-methylpyrazole. *CYP*1A1/2 are the main enzymes responsible for the metabolism and activation of polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene and benzo[*a*]pyrene [30]. *CYP*1A1 has been demonstrated to be inducible by ethanol [43]. Norharman was shown to inhibit the metabolism of the *CYP*1A1 substrate benzo[*a*]pyrene by rat liver microsomes. It inhibits the conversion of hydrophobic to hydrophilic metabolites, leading to the formation of the strong mutagen 7,8-dihydroxybenzo[*a*]pyrene, which was increased 10-fold after coincubation with norharman [44].

Displacement studies with coumarine, a ligand for *CYP*2A6 [45], were limited by its poor solubility in incubation buffer. This *CYP* isozyme has been reported to take part in the activation of nitrosamine derivatives as well [46]. *CYP*2A6 constitutes only a minor form of the cytochrome P450s, i.e. about 1% of the total *CYP* content in human liver with high variation [45]. Therefore, its role in the metabolic activation of carcinogens is rather unclear.

Ligands of *CYP*2B1/2 trans-stilbene-oxide [30] and *CYP*2D6 chlorpromazine [47] and quinidine [48], which were suggested to have an affinity to the [^{3}H]norharman binding sites at microsomal rat liver membranes in screening tests, failed to displace [^{3}H]norharman significantly in more elaborate displacement experiments.

Displacement of specific [^{3}H]norharman binding in rat liver membranes by ligands of different *CYP* families and subfamilies indicate that the biphasic inhibition curves of the *CYP*2E1 ligands and the 6-substituted BCs result from binding to other *CYP* enzymes with lesser affinity (e.g. *CYP*1A1/2 and *CYP*2A6). Furthermore, binding to other cell proteins cannot be excluded by our membrane preparation procedure. Pyrazole and 4-methylpyrazole are both used to study alcohol dehydrogenase (EC 1.1.1.1), but this enzyme is located in the cytoplasm [49] and was therefore not present in the incubation mixture.

Our *in vivo* experiments are consistent with the conclusion that [^{3}H]norharman binds with high affinity to the ethanol-inducible *CYP*2E1. Chronic treatment with ethanol caused an increase of norharman in blood plasma, suggesting an interaction of the metabolism of ethanol and norharman. This notion was further supported by the finding that 28 days after ethanol withdrawal, the norharman levels were reduced, possibly due to the increased metabolism of norharman. Why these levels were not reduced 8 days after ethanol withdrawal remains to be elucidated. It is interesting to note that no significant change in harman blood plasma levels were found, which is consistent with our *in vitro* findings that harman does not bind to the [^{3}H]norharman binding sites in rat liver or to the human lymphoblast-expressed rat *CYP*2E1 enzyme.

In conclusion, data from the present study further support the notion that norharman binds with high affinity to those *CYP* enzymes (*CYP*2E1, *CYP*1A1/2, and *CYP*2A6) which take part in the activation of many promutagens to mutagens and carcinogens. Because BCs are found in cigarette smoke and foodstuff and have also been demonstrated to be elevated in alcoholic patients, who run a well-known higher risk of cancer due to alcohol abuse, binding and therefore possibly interference with *CYP* enzymes contribute to a further understanding of norharman's comutagenic action.

The authors thank H. Damm and S. Lutter for expert technical assistance. We would also like to thank Prof. J. Lehmann (University of Bonn, Germany) and Prof. M. A. Collins (Loyola University, IL) for their gifts of β-carboline derivatives. The study was supported by the Deutsche Forschungsgemeinschaft (AZ He 916/7-2).

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