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Isolation and identification of two [^{3}H]norharman-([^{3}H]β-carboline)-binding proteins from rat liver

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Abstract

Norharman (9H-pyrido-[3,4-b]indol) represents a member of the mammalian alkaloids with the group name β-carbolines. In mammals, it exhibits psychotropic and co-mutagenic actions. Highly specific [^{3}H]norharman binding sites have been detected in the liver of rats (B_{max}: 11 pmol mg^{-1} protein; K_D: lower nanomolar range). Two [^{3}H]norharman binding proteins with apparent molecular masses of 60 and 80 kDa (SDS–PAGE) were isolated from rat liver crude membrane fraction and identified as the enzyme carboxylesterase (EC 3.1.1.1; 60 kDa) and the stress protein glucose-regulated protein 78 (GRP78; 78 kDa). Possible functional consequences of the interaction of norharman with these two proteins are discussed.

Keywords: Norharman; β-Carboline; Proteins

1. Introduction

The term β-carboline (harmala-alkaloids; BC) characterizes a group of indole alkaloids, possessing a tricyclic pyrido[3,4-b]indol basic structure including two heterocyclic nitrogen atoms (Fig. 1a). Many BCs exhibit psychotropic, mainly hallucinogenic, actions, which were known for some decades. Based on this knowledge, plants containing BCs have been used by some tribes for the preparation of ritual beverages [1,2]. Concerning norharman, it has been shown that a rapid increase of natural norharman levels in patients which occurs during traumatic stress disorders is associated with hallucinations [3].

Norharman (9H-pyrido-[3,4-b]indol) is the structurally simplest fully aromatic BC which lacks any substituents at the ring system and has therefore been designated β-carboline. Its occurrence in nature has been described in plants, fungi, foodstuff and tobacco smoke, as well as in mammals, including men [4,5]. In rodents, norharman induces pro-conflict behaviour, tonic-clonic convulsions and locomotor activation [6,7]. In addition, norharman exhibits co-mutagenicity (for review: [8]) and the β-carbolinium ion may contribute to the neurotoxic processes in the pathogenesis of Parkinson's disease [9–11].

Concerning drug dependence and alcoholism, BCs are of great interest. Regarding animal experiments, they increase voluntary ethanol intake in rats [12]. Norharman activates the mesolimbic dopaminergic reward system as indicated by a concentration dependent increase of extraneuronal dopamine levels suggesting a role in psychological dependence [13].

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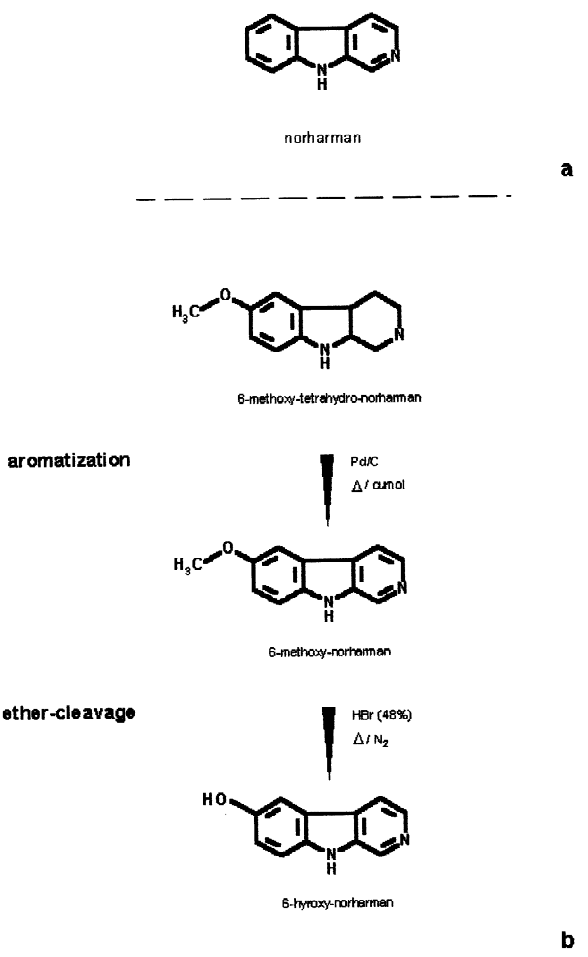

Fig. 1. (a) Chemical structure of β-carboline; synonymous: *norharman*. (b) Reaction scheme of the synthesis of the affinity ligand 6-hydroxy-norharman.

Clinical studies showed about 3-fold higher norharman levels in blood and urine of alcohol dependent patients, compared to controls. A connection between norharman and the development and/or manifestation of alcoholism is very well conceivable [14,15]. Investigating the cellular basis of those clinical findings, a receptor or receptor-like molecule was searched through which norharman could exert its pharmacological actions in the mammalian body. Specific high affinity binding sites for tritium-labelled norharman have been detected in various organs of the rat, namely in brain, adrenal gland and liver. The pharmacological profile and distribution pattern of brain binding sites could not be correlated to those of any known receptor [16–18]. A biosynthetic pathway for the endogenous formation of β-carbolines in mammals has been proposed, which up to now, could only be verified in parts [5,6]. Knowledge of the nature of liver binding proteins and the role they play in mammalian organism could lead to further insights into the function of the natural hormone-like alkaloid norharman. Furthermore, the previously described elevated blood concentrations of norharman could be of great importance for secondary pathological damages, contributing to the high incidence of carcinomas and possibly liver cirrhosis in alcoholic patients.

This study was aimed at identifying further cellular targets of norharman to better understand the mechanisms by which this compound exerts its multiple actions. The density of [^{3}H]norharman binding sites was highest in liver membranes showing a similar pharmacological profile and binding characteristics as the low-affinity brain binding sites (K_D: 46 n*M*, B_{max}: 15 pmol mg^{-1} protein). In order to purify and identify specific [^{3}H]norharman binding sites and to keep the number of animals to be killed as small as possible, we decided to work with rat liver. The liver seemed to provide a suited source for preparative purposes because of the aforementioned similarities between the brain and the liver binding sites and its higher quantity of binding sites per organ. Using FPLC-column chromatography, applying the synthesized affinity resin ''norharman sepharose'', two norharman binding proteins could be isolated from rat liver (Fig. 2). Internal N-terminal amino acid sequencing of tryptic peptides and computer-assisted sequence analysis identified them as the enzyme carboxylesterase (EC 3.1.1.1; 60 kDa) and the glucose-regulated protein 78 (GRP78/BiP; 78 kDa), which belongs to the large family of heat-shock proteins.

Possible functional consequences of the interaction of norharman with these two proteins are discussed.

2. Experimental

2.1. Materials

2.1.1. Animals

The livers of 3–6-month-old male Wistar rats (Hagemann, Lippische Versuchstierzucht, Extertal, Germany) were utilized. The animals were housed in

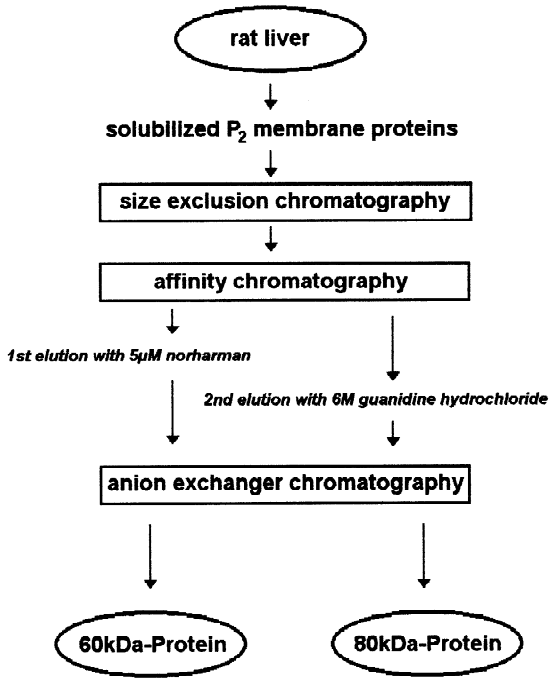

Fig. 2. Purification scheme of norharman binding proteins.

groups of four and kept in an animal room under a 12:12 h light:dark cycle, with standard diet (Altromin 1324) and water ad libitum. The experiments were performed in accordance with the legal requirements of the national authority.

2.1.2. Buffers

MOPS (3-*N*-morpholinopropanesulfonic acid) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-propane-1-sulfonic acid): Carl Roth KG (Karlsruhe, Germany); TRIS (2-amino-2-hydroxymethylpropane-1,3-diol): Sigma (Deisenhofen, Germany).

2.1.3. Radioligand binding assay

[^{3}H]norharman: custom labelled by Amersham International plc (Buckinghamshire, UK), specific activity: 25–50 Ci mmol^{-1}; 12-well-cell harvester: Brandel Inc. (Gaitersburg, USA); glassfiber membrane filters ''Whatman GF/B'': Dunn (Asbach, Germany); liquid scintillation solution ''Ultima Gold'', 1900CA Tri Carb Liquid Szintillation Analyser: Packard (Groningen, Netherlands); polyethyleneglycol; Merck (Darmstadt, Germany); polyethyleneimine: Sigma (Deisenhofen, Germany).

2.1.4. Chromatography

(i) FPLC: Econo-system: BioRad (Munich, Germany); Ultrogel AcA44: Serva (Heidelberg, Germany); Q-Sepharose Fast Flow and Epoxy-activated Sepharose 6B: Pharmacia (Freiburg, Germany); 6-methoxy-tetrahydronorharman: Aldrich (Steinheim, Germany). (ii) HPLC: Hewlett-Packard chromatograph type 1090 with fluorescence detector type 1046A; Intersil C_8: VDS Optilab (Berlin, Germany).

2.1.5. Electrophoresis

Acrylamide, bisacrylamide, SDS, β-mercaptoethanol: Sigma (Deisenhofen, Germany); molecular mass standards for SDS–PAGE, Serva Blue R: Serva (Heidelberg, Germany); ammoniumpersulphate: BioRad (Munich, Germany); TEMED: Carl Roth KG (Karlsruhe, Germany).

2.1.6. Protein determination

BCA, $CuSO_4 \times 5H_2O$ (4%): Sigma (Deisenhofen, Germany); BSA: Serva (Heidelberg, Germany).

All other chemicals and reagents used were of at least p.a. quality.

2.2. Preparation of rat liver

Immediately after decapitation, livers were dissected off the animals and shock frozen in liquid nitrogen. They were stored at −80 °C. Pilot experiments revealed that storage of 6 months was possible without loss of [^{3}H]norharman binding activity.

2.3. Preparation of P_2 membrane proteins

Isolation of P_2 membrane proteins was performed according to [18] with minor modifications. In brief, 3–6 g rat liver were homogenized in 10 volumes of cold (4 °C) 25 m*M* K-MOPS, pH 7.4, using a glass-teflon homgenizer (10 strokes). The homogenate was centrifuged for 10 min at 1000 *g* (4 °C). The resulting supernatant was centrifuged for 30 min at 23 000 *g* (4 °C). The resulting pellet (P_2 crude cell membrane fraction) was frozen at −80 °C overnight and homogenized in 10 volumes of cold (4 °C) 25 m*M* K-MOPS–10 m*M* CHAPS, pH 7.4. Solubilization was performed by stirring the homogenate for 60 min at 0 °C (ice–water-bath), avoiding foam-generation. After the third centrifugation (60 min at 105 000 *g*, 4 °C) the resulting supernatant, containing

the P_2 crude cell membrane proteins, was frozen and stored at −80 °C in 5 and 10 ml aliquots.

2.4. [^{3}H]norharman binding assay

Radioligand binding assay was performed according to [18] with minor modifications: In the case of column-screening, routinely 300 μl of fractions of interest were tested, independent of the protein content. Specific binding (dpm–μg protein) was calculated after protein determination. In brief, 300 μl of the column fraction to be tested were placed into a test tube (0 °C, ice–water-bath), 100 μl 2.5 μ*M* unlabelled norharman (determination of unspecific ligand binding; 500 n*M* per test tube) or buffer (determination of total ligand binding) and 100 μl of cold buffer were added. The incubation was started by adding 100 μl 20 n*M* [^{3}H]norharman (4 n*M* per test tube). The tubes were vortexed intensively and incubated at 0 °C for 2 h (ice–water-bath). At least duplicates were analysed. Binding was terminated by precipitating the protein after addition of 1 ml ice cold 30% w/v polyethyleneglycol in 25 m*M* K-MOPS, pH 7.4, for 1.5 min. Bound [^{3}H]norharman was separated by filtration through glassfiber membranes, preincubated for at least 2 h in 0.1% w/v polyethyleneimine, using a 12-well cell harvester. Test tubes were rinsed twice with about 3 ml ice cold 50 m*M* Tris–HCl, pH 7.4, containing 8.5% w/v polyethyleneglycol. Filter membranes were shaken with 4 ml liquid scintillation solution for 1 h at room temperature. After a resting period of another hour, radioactivity was counted for 5 min each. Specific radioligand binding was calculated by subtraction of unspecific binding (determined with 500 n*M* unlabelled norharman as displacer) from total ligand binding.

2.5. Synthesis of the affinity chromatography resin "norharman-sepharose"

2.5.1. Synthesis of the affinity ligand 6-hydroxy-norharman

(a) *Aromatization*: 610 mg 6-methoxy-tetrahydro-norharman were boiled for 30 min with freshly distilled cumol (isopropylbenzene) (1 ml–40 mg) at 152 °C, using Pd–C-catalysator (0.5–1 mg substrate). The reaction mixture was filtered hot and the filter supernatant was washed with ethanol. The organic phases were combined and the solvent was evaporated. The remaining residue was re-crystallized out of toluol. Product purity and identity was confirmed by thin layer-chromatography and melting point-determination.

(b) *Ether-cleavage*: 410 mg 6-methoxy-norharman in total were boiled with 1.4 g HBr (48%) for 2.5 h under nitrogen atmosphere. The cooled down reaction mixture was poured onto 20 g of ice and neutralized using 6 N NaOH. The precipitate was separated by filtration and crystallized out of H_2O. Product purity and identity was confirmed by thin layer-chromatography, melting point-determination and IR, as well as NMR chromatography.

2.5.2. Preparation of the affinity-matrix "norharman-sepharose"

A total of 48 mg (261 μmol) 6-hydroxy-norharman were dissolved in 20 ml 10 m*M* NaOH–dioxan (1:1; v/v). Subsequently, 9 ml Epoxy-activated Sepharose 6B (pre-swollen in H_2O) were added. This mixture was rotated on an upside-down-shaker for 60 h at room temperature. Aliquots of the supernatant of the ligand-matrix suspension were taken at the start of and several times during the 60 h run of the coupling reaction. Progress of the ligand coupling was monitored: (i) semi-quantitative by running 10 μl aliquots on a thin layer-chromatography plate (containing fluorescence indicator; solvent: dichloromethane–methanol = 9/1) and comparing spot size and intensity; and (ii) by quantification of 6-hydroxy-norharman in 50 μl aliquots via reversed-phase HPLC (pre-column: 1.5 cm; column: 250×4.6 mm; packing: Intersil C_8; solvent: 10% acetonitrile, 90% ammonium acetate buffer; flow: 1.5 ml min^{-1}). The resulting affinity resin was harvested by filtration and, to block uncoupled epoxy-groups, incubated in 1 *M* ethanolamine for 4 h at room temperature (upside-down-shaker). Then again, the resin was separated from the fluid by filtration and washed with 150 ml 5 m*M* NaOH–50% dioxan, 100 ml 0.1 *M* $KHCO_3$ and 100 ml CH_3COOK. Afterwards, the matrix was filled into a chromatography column (d: 1.5 cm; l: 10 cm) up to a height of 9 cm, representing 16 ml gelbed volume, connected to the chromatography system and equilibrated with 100 ml

25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4, at a flow-rate of 0.1 ml min^{-1}.

2.6. Identification of the [^{3}H]norharman binding proteins

Isolated proteins were eluted out of SDS-gels or electroblotted onto PVDF membranes. Tryptic peptides were separated using HPLC chromatography. The most prominent peptides were identified applying internal N-terminal protein sequencing and computer-aided comparison of the fragment sequences to those of already known proteins in common sequence data libraries (WITA GmbH, Teltow, Germany).

3. Conditions

3.1. Size exclusion chromatography

Chromatography was performed at 4 °C, using Ultrogel AcA 44 in 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4. Column dimensions: d = 2.5 cm, h = 115 cm (V_{gel} = 565 ml). 81–90 mg protein in 18–20 ml of liver solubilisate were used for a single column run at a flow-rate of 0.16 ml min^{-1}. V_o was determined to be 150 ml (using blue dextran) and was discarded. Eighty fractions of 5 ml each were collected. Aliquots from every second fraction were tested for radioligand binding, protein content and SDS–PAGE-pattern. Remaining fractions were frozen at −80 °C until further analysis.

3.2. Affinity chromatography

Size exclusion chromatography fractions with highest [^{3}H]norharman binding activity were detected around fraction numbers 10–30 (±2), representing 100±25 ml (11.5±2.9 mg protein) starting material for affinity chromatography. Pooled fractions were incubated with the affinity resin (9 ml; suspended in 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4) for 2 h in a cold room (4 °C), either gently shaken or slowly stirred in an erlenmeyer flask in a manner that all the matrix particles were in constant movement. After this incubation period, the resin was filled into a chromatography column (d: 1.5 cm; h: 10 cm), which was then connected to the chromatography system and washed with 30 ml 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4 at a flow-rate of 1 ml min^{-1}. Washing was followed by the first elution step: the resin again was filled into an erlenmeyer flask and gently shaken overnight (4 °C) with 20 ml of 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4, containing 5 μ*M* norharman. After filling the matrix into the column and collecting the flow-through, a second elution was performed with 200 ml 5 μ*M* norharman (dissolved in 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4) at a flow-rate of 0.1 ml min^{-1}. Fraction size was set to 5 ml. Aliquots of 500 μl were collected from every second fraction for SDS–PAGE analysis. The remaining fractions were frozen at −80 °C until further chromatography and analysis.

3.3. Ion-exchange chromatography

Chromatography was performed at 4 °C, using the anion exchanger resin Q-Sepharose Fast Flow in 25 m*M* Tris–HCl–5 m*M* CHAPS, pH 7.6. Column dimensions: d = 1.5 cm, h = 7.5 cm (V_{gel} = 13 ml). Standard flow-rate for elution was set to 1 ml min^{-1}, fraction volume to 2.5 ml. Elution of proteins was performed using a linear or step-wise salt gradient with KCl in buffer (25 m*M* Tris–HCl–5 m*M* CHAPS, pH 7.6). Gradient generation was produced automatically by the chromatography system. Sample analysis was performed according to the procedure described for size exclusion chromatography.

3.4. Electrophoresis

Electrophoresis of proteins under denaturing conditions (SDS-PAGE) was performed according to Laemmli [19] in a vertical gel system, using an acrylamide gradient ranging from 10–15% (4% for stacking gel). Initial voltage was set to 130 V, final values usually not exceeding 220 V.

3.5. Protein determination

Protein determination was performed according to the method of Smith et al. [20] using bicinchoninic acid as reagent and bovine serum albumin as a standard.

4. Results

4.1. Synthesis of the affinity chromatography resin "norharman-sepharose"

4.1.1. Yield and identity of the ligand 6-hydroxy-norharman

Fig. 1b shows the reaction scheme of 6-hydroxy-norharman synthesis.

In a first reaction, 410 mg (2.100 mmol) of the intermediate compound 6-methoxy-norharman were gained by the aromatisation of 610 mg (3.062 mmol) 6-methoxy-tetrahydronorharman. This represents an efficiency of 68.6%.

In a second reaction, ether-cleavage of 6-methoxy-norharman resulted in a total amount of 295 mg (1.628 mmol) of 6-hydroxy-norharman. This represents an effiency of 77.5%. The identification of the affinity ligand was performed by comparing the infrared (IR) and nuclear magnetic resonance (NMR) spectra (Fig. 3) with those described in the first report [21]. The spectra unequivocally showed the same compound, the substituted β-carboline 6-hydroxy-norharman.

4.1.2. Coupling efficiency of the affinity matrix

At the start of and several times during the 60 h run of the coupling reaction between 6-hydroxy-norharman and the matrix, Epoxy-activated Sepharose 6B aliquots of the supernatant were analyzed semi-quantitative utilizing TLC plates. It was shown that at $t = 23$ h about 60% and at $t = 60$ h about 40% of the ligand still were present unchanged. A higher coupling efficiency was not observed at longer reaction times, even when 50 μl aliquots of the reaction supernatant at the corresponding time points were analysed quantitatively by HPLC analysis (data not shown): unreacted 6-hydroxy-norharman in the supernatant of the starting solution eluted at 6.2 min (authentic norharman eluted at $t = 6.714$ min) and the peak showed an integrated signal of 48.08 area%. Taking this value as 100%, the portion of ligand remaining in the supernatant of the coupling solution after 60 h of reaction was reduced to 38% (18.46 area%).

The starting reaction mixture of ligand and activated gel matrix contained 29 μmol ligand per 1 ml wet gel. According to HPLC measurements 62% of ligand was coupled to the matrix. Therefore, a coupling efficiency of 18 μmol ligand−1 ml gel was achieved. The instruction manual of Epoxy-activated Sepharose 6B described that a maximum number of 40 μmoles−1 ml wet gel of activated oxirane-groups were present in the matrix. According to this, the resulting coupling efficiency represents a value of 45%. Therefore, the coupling reaction of 6-hydroxy-norharman to Epoxy-activated Sepharose 6B was quite optimal since, according to the manufacturers instructions, a coupling efficiency of over 50% would not result in improved protein purification, due to very tightly bound proteins that sometimes cannot be eluted without denaturation.

4.2. Isolation of specific [^{3}H]norharman binding proteins

4.2.1. Size exclusion chromatography

Using size exclusion chromatography as the first purification step a large number of proteins could be separated from those fractions containing specific [^{3}H]norharman radioligand binding capacity. The elution profile of a typical run is shown in Fig. 4. The majority of proteins clearly elute from the column after the peak of high affinity binding proteins has passed. The binding proteins are in the molecular mass range of about 400 kDa (determined by running the column with marker proteins). This seemingly high apparent molecular mass is not unusual since the elution buffer contains 5 m*M* CHAPS. The [^{3}H]norharman binding-protein(s) are likely inserted into detergent micelles, together with other proteins. The pooled fractions from size exclusion chromatography showed specific [^{3}H]norharman binding of 500 dpm mg^{-1} protein. Crude P_2 membrane solubilisate (starting material) showed specific binding of 113 dpm mg^{-1} protein. Considering the extent of radioligand binding degradation, due to the duration of the purification procedure, which was calculated from former experiments, the enrichment of specific binding after size exclusion chromatography resulted in a factor of 4.4. This is close to the theoretical maximum of 5 for this method.

4.2.2. Affinity chromatography

(a) *Isolation of a protein with an apparent molecular mass of 60 kDa*. The pooled fractions from size

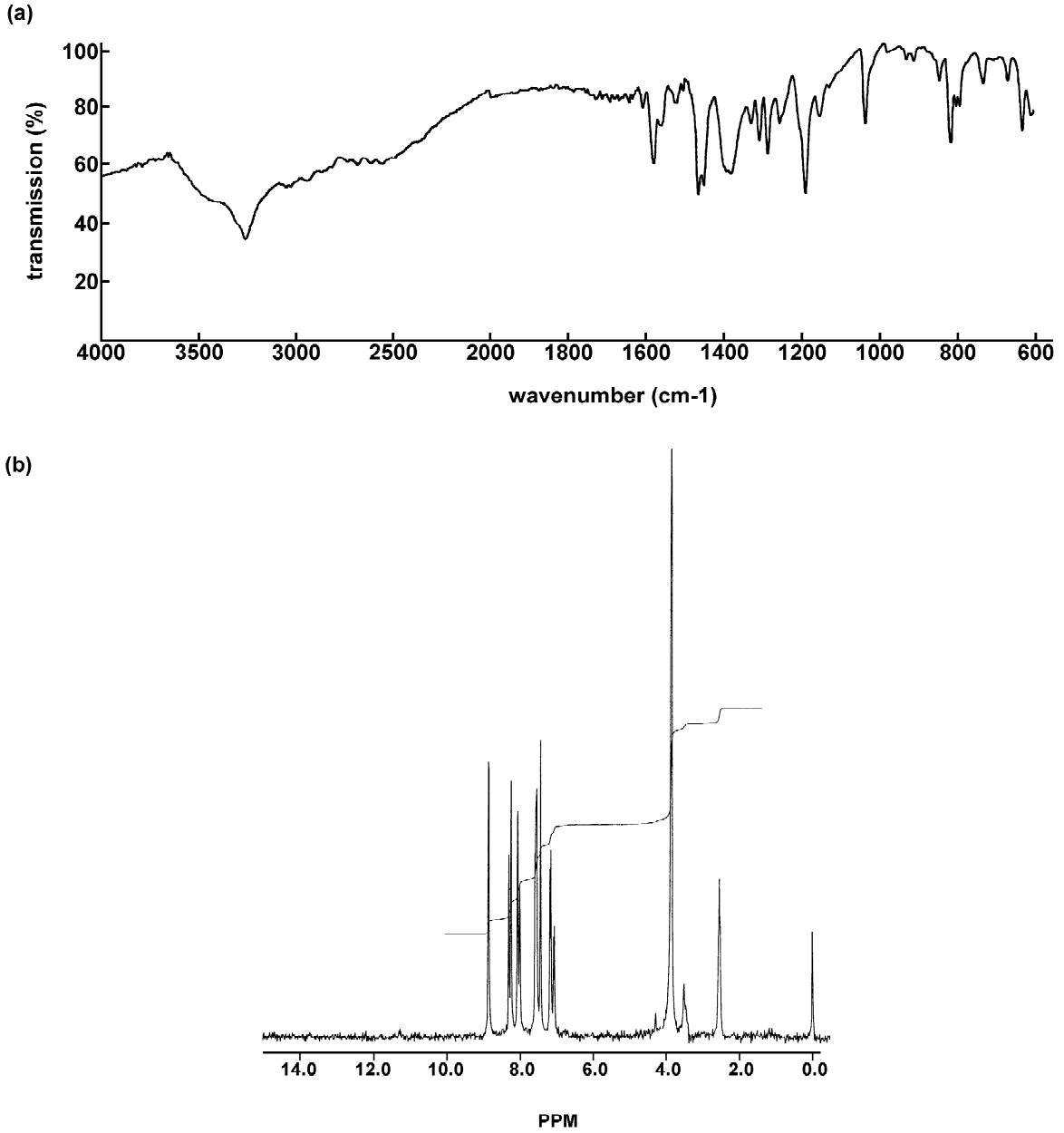

Fig. 3. Identification of synthezised 6-OH-norharman. (a) Infrared (IR) spectrum of 6-OH-norharman: in comparison to the starting material 6-CH_3-O-norharman the characteristic OH-band between wavenumbers 3500^{-1} and 3000^{-1} is visual and the CH_3-O-bands around wavenumber 2500^{-1} have disappeared. (b) Nuclear magnetic resonance (NMR) spectrum of synthezised 6-OH-norharman.

exclusion chromatography containing high affinity radioligand binding were incubated with ''norharman-sepharose''. In order to get the highest matrix–protein interaction possible, incubation was performed according to radioligand binding assay conditions. The elution of bound [^{3}H]norharman binding proteins from the column under ''non-specific'' conditions (using salt gradients instead of norharman

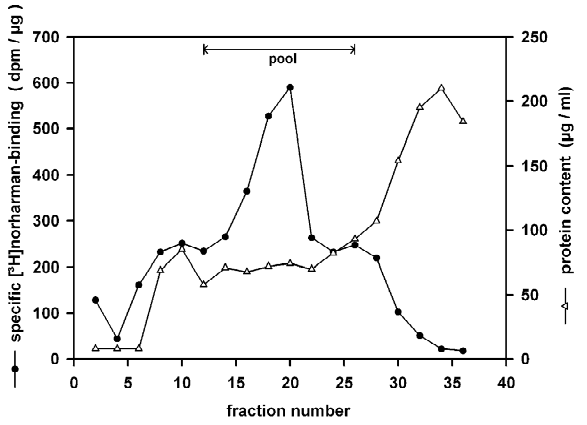

Fig. 4. Elution profile of a representative size exclusion chromatography run. Solubilized P_2-membrane proteins were run on Ultrogel AcA44 (column: 2.5×115 cm; fraction size: 5 ml; elution: 0.16 ml min^{-1}). The exclusion limit of the column V_0 was determined as 150 ml using blue dextran and was discarded; *circles*: specific [^{3}H]norharman binding (dpm–µg protein); *triangles*: protein content (µg ml^{-1}). For further chromatography on 6-OH-norharman-sepharose every fraction with a minimum of 10^3 dpm–300 µl was used.

displacers for protein elution) showed no separation efficiency (data not shown). Therefore, specific elution with a high affinity binding ligand had to be performed. Since no specific protein ligand with higher binding affinity than norharman is known, norharman itself had to be used for protein elution. Association and dissociation kinetics of specific [^{3}H]norharman binding revealed that association (under the conditions used in the binding assay) is nearly completed after 2 h and dissociation (using micromolar concentrations of unlabelled norharman) seemed to occur in a biphasic manner and very slowly. The first displacement plateau was seen around 5 h of incubation and total dissociation was completed only after more than 16 h (Greube, former experiments). Therefore, elution with unlabelled norhaman was performed very slowly. Following extensive washing of the affinity resin, specific elution with 5 µ*M* unlabelled norharman at a low flow-rate (0.1 ml min^{-1}) resulted in the detection of a single protein band with a molecular mass of approximately 60 kDa, as assessed by SDS–PAGE.

(b) *Elution of a second protein with high [^{3}H]norharman binding affinity using high salt and denaturing conditions*. After specific elution was completed, the column routinely was washed with 1 *M* KCl (in 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4). Under these conditions a protein fraction was eluted which still showed a high capacity of specific [^{3}H]norharman binding, pointing to the existence of another norharman binding protein which binds more tightly to the affinity matrix. Elution of the column with two column volumes of 6 *M* guanidine hydrochloride (in 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4, at a flow-rate of 0.5 ml min^{-1}) resulted in the detection of a single protein with an apparent molecular mass of approximately 80 kDa, as determined by SDS–PAGE. It was concluded that this protein could represent a second [^{3}H]norharman binding protein of higher affinity than the 60 kDa protein. Dialysis of the pooled fractions containing the 80 kDa protein (Σ: 7.5 ml) was performed two times (18–20 h) against 25 m*M* K-MOPS, pH 7.4, at 4 °C to renature the protein. Aliquots of the dialysed solution showed high specific [^{3}H]norharman binding of 1239 dpm–300 µl.

4.2.3. Ion exchange chromatography

(a) *Enrichment of a protein with an apparent molecular mass of 60 kDa*. The 60 kDa protein isolated by affinity chromatography was highly diluted in a buffer containing 5 µ*M* norharman. Under these conditions it was impossible to detect specific radioligand binding, bearing in mind that the IC_{50} for norharman displacement is in the lower nanomolar range ($K_D = 5\times10^{-9}$ n*M*; [18]). In order to concentrate the diluted sample and to replace sample buffer by one suited for radioligand binding experiments, affinity chromatography was followed by ion-exchange chromatography. Preliminary experiments had shown that [^{3}H]norharman binding proteins could be bound and eluted by a salt gradient using an anion exchanger resin. Fig. 5 shows the elution profile of a typical anion exchanger run where the protein of interest is eluted in a sharp peak at a salt concentration of approximately 0.6 *M* KCl. The fraction of the highest protein content exhibited specific [^{3}H]norharman binding of 431 dpm–300 µl.

Since the binding degradation during the purification process and a remaining norharman concentration in ion exchanger fractions of about 10–15 n*M* (HPLC measurement), ''real'' radioligand binding was calculated, taking all the factors into account

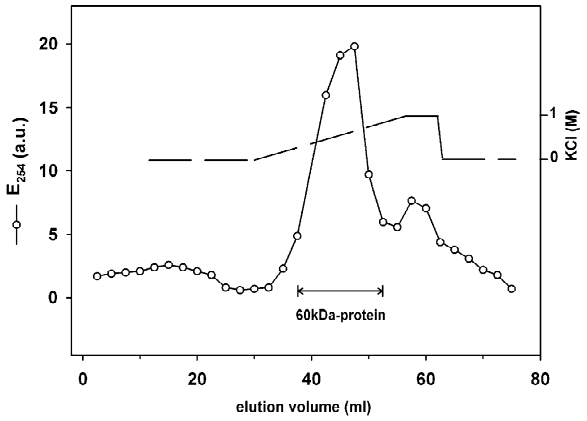

Fig. 5. Isolation of the 60 kDa [^{3}H]norharman binding protein by anion exchange chromatography using a continuous salt gradient. The pooled fractions of the specific elution of 6-OH-norharman-sepharose (using 5 μ*M* norharman as eluent) were run on Q-Sepharose Fast Flow (column: 1×7.5 cm; fraction size: 2.5 ml; elution: 1 ml min^{-1}) in order to concentrate eluted protein(s) and to wash out norharman. Proteins were eluted using a continuous KCl-gradient between 0 and 1 *M*. Fractions of highest protein content (E_{254} above 5; *circles*) were analysed according to the protein pattern (SDS–PAGE) and [^{3}H]norharman binding.

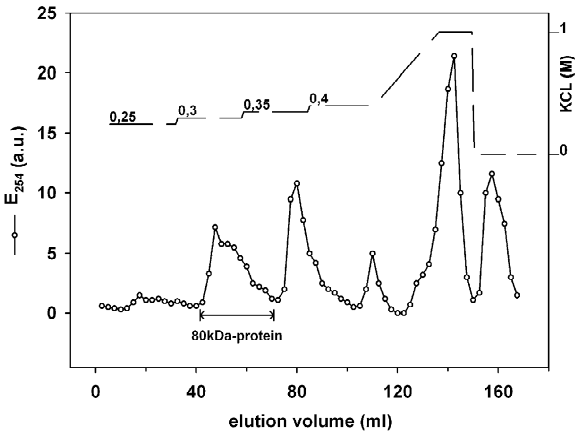

Fig. 6. Isolation of the 80 kDa [^{3}H]norharman binding protein by anion exchange chromatography using a step-wise salt gradient. The second (high salt) elution pool of 6-OH-norharman-chromatography (using 1 *M* KCl as eluent) was also run on Q-Sepharose Fast Flow (column: 1×7.5 cm; fraction size: 2.5 ml; elution: 1 ml min^{-1}) but here proteins were separated using a carefully generated stepwise KCl-gradient between 0 and 0.4 *M*. Fractions of highest protein content (E_{254} above background; *circles*) were analysed according to the protein pattern (SDS–PAGE) and [^{3}H]norharman binding.

contributing to lowered binding (according to protein handling between and during the different purification steps like freeze–thaw-cycles and maintenance at 4 °C).The isolated 60 kDa protein possessed a maximum specific [^{3}H]norharman binding capacity of 4300 dpm mg^{-1} protein. Compared with the results of size exclusion chromatography (500 dpm mg^{-1}) this represents an enrichment of 8.6 times, compared to the starting material an enrichment of 38-fold.

(b) *Isolation of a protein with an apparent molecular mass of 80 kDa*. The 80 kDa protein, which could be eluted from the affinity matrix using denaturing conditions, could be separated from remaining traces of the 60 kDa protein by a stepwise salt elution of the anion exchanger matrix, using 0.05 *M* KCl steps. At a salt concentration of 0.3 *M* KCl the protein eluted from the column, regardless of the starting material used for the run (size exclusion chromatography pool, high-salt pool following affinity chromatography, ion exchanger pre-run pool with continuous gradient, where the 60 kDa protein could also be eluted). Every fraction eluting at 0.3 *M* salt showed a main protein band representing the 80 kDa protein. Minor contaminant patterns varied with starting material. A typical elution profile is shown in Fig. 6.

(c) *The 80 kDa protein represents a second [^{3}H]norharman binding protein*. The isolated 80 kDa protein showed surprisingly high stability of [^{3}H]norharman binding characteristics: although some cycles of re-chromatography (including freeze–thaw-cycles and elongated times at 4 °C) had been performed, specific [^{3}H]norharman binding of samples with very low protein content remained clearly detectable. In addition, another experiment underlines this stability: the 80 kDa protein band was cut out of an SDS-gel, the gel slice was crushed with a glass pistil and the protein was eluted by incubation of the crushed gel slice in 500 μl 6 *M* guanidine hydrochloride (in 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4) at 4 °C for 3 days. Following filtration, the solution was dialysed against 25 m*M* K-MOPS–5 m*M* CHAPS, pH 7.4 (two times, overnight, 4 °C). Aliquots of this solution showed remarkably high radioligand binding of 343 dpm μg^{-1} protein. To exclude artificial results, the experiment was repeated two times, yielding similar results. In addi-

tion, for negative control, marker protein bands (BSA, ovalbumin) have been treated the same way, showing no radioligand binding at all.

Based on the aforementioned experiments, specific [^{3}H]norharman binding can only be regarded qualitatively. It is not known, in what order of magnitude, the electrophoretic separation and the elution procedure reduced radioligand binding capacity compared to starting material and up to what percentage renaturation occurred. Nevertheless, the existence of stable, specific [^{3}H]norharman binding to the 80 kDa protein cannot be called into question.

The appearance of the two isolated [^{3}H]norhaman binding proteins on SDS-gels is shown in Fig. 7.

4.3. Identification of the [^{3}H]norharman binding proteins

The 60 and 80 kDa proteins have been identified using internal N-terminal protein sequencing and computer-aided comparison of the fragment sequences to those of already known proteins. The 60 kDa protein that was eluted from the anion exchanger matrix had been pooled and the whole protein from one run was electroblotted onto PVDF membranes according to [22]. For identification of the apparent 80 kDa protein the coomassie stained and intensively water soaked (overnight, 4 °C, storing the gel in water) electrophoresis gel of concentrated anion exchanger fractions was used.

4.3.1. The 60 kDa protein

After proteolytic cleavage of the blotted protein using trypsin, resulting peptides were separated using HPLC-chromatography. The peptides were numbered according to their order of elution. The most prominent three peptides were sequenced in a length of about 10 amino acids, starting at the N-terminus (WITA GmbH, Teltow, Germany). Computer-aided sequence comparison (using the GenBank library) showed for all three peptides 100% identity with the rat protein *carboxylesterase* (EC 3.1.1.1). Fig. 8a shows the sequences of the peptides analysed in comparison to the published sequences of the rat protein.

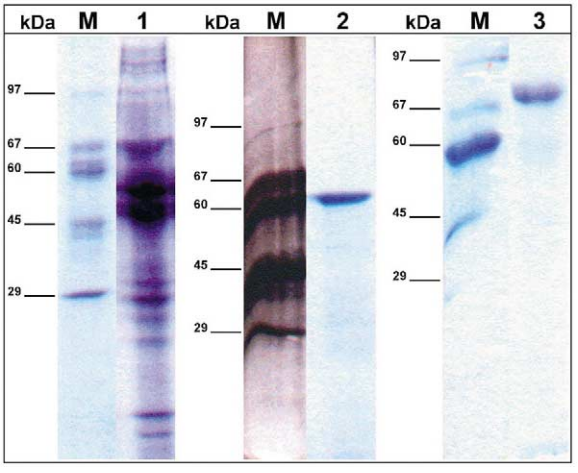

Fig. 7. SDS–PAGE patterns of different purification steps. *Lane 1*: gel chromatography-pool; *lane 2*: 60 kDa-protein (peak of anion exchanger chromatography with continuous salt gradient elution); *lane 3*: 80 kDa-protein (0.3 *M* KCl-peak of anion exchanger chromatography with stepwise salt gradient elution); *M*: molecular mass markers of 97, 67, 60, 45 and 29 kDa.

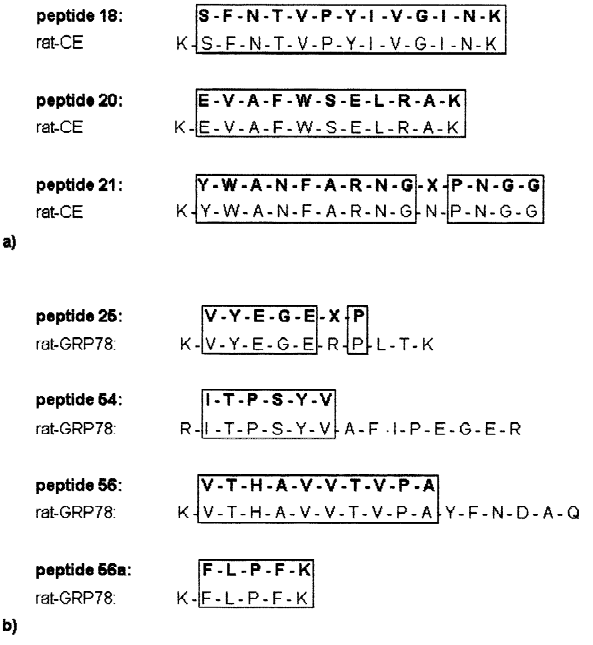

Fig. 8. Sequence-comparison of isolated [^{3}H]norharman binding protein peptides with the corresponding fragments of rat proteins. (a) 60 kDa protein versus carboxylesterase (CE). (b) 80 kDa protein versus glucose-regulated protein 78 (GRP78).

4.3.2. The 80 kDa protein

The protein was analysed as described for the 60 kDa protein with the only exception of using a different starting material: due to the low protein content, the blotting step was omitted in order to minimize loss of protein. Furthermore, irreversible changes of proteins due to the blotting procedure and attachment to the blotting membrane are avoided. Using the proteolytically cleaved protein, again three peptides were sequenced successfully. Computer-aided sequence comparison with known protein sequences (GenBank) showed for all three peptides 100% identity with the rat protein *glucose-regulated protein 78* (GRP78/BiP). Fig. 8b shows the sequences of the proteins analysed in comparison to the published sequences of the rat protein.

5. Discussion

Norharman (9H-pyrido-[3,4-b]indol) represents a member of the mammalian alkaloids exhibiting psychotropic and co-mutagenic effects. Its actions on the molecular level are only known in part. For example, it inhibits the enzyme monoamineoxidase subtype B (MAO-B), it activates pertussis toxin-sensitive small GTP binding proteins in a receptor-independent manner [23,24] where it facilitates inositol phosphate accumulation [25] and binds to cytochrome P450 2E1 [26]. Distinct and high-affinity [^{3}H]norharman binding sites have been detected in various organs of the rat, including brain and liver. Their distribution patterns in brain could not be correlated with those of any known receptor [16,27].

In this study, by applying the specially synthesized affinity matrix ''norharman-sepharose'', two [^{3}H]norharman binding proteins with apparent molecular masses of 60 and 80 kDa (SDS–PAGE) could be isolated from rat liver P_2 membranes. These [^{3}H]norharman binding proteins have been identified as the enzyme carboxylesterase (EC 3.1.1.1; 60 kDa) and the stress-protein glucose-regulated protein 78 (GRP78; 78 kDa). Possible functional consequences of the interaction of norharman with these two proteins with special regard to ethanol metabolism and drug abuse will be discussed.

Carboxylesterases are a mammalian multigene family of detoxifying serine esterases, which catalyse hydrolysis of a wide range of xenobiotic carboxylesters, thioesters or aromatic amide groups including a wide variety of ester-type drugs [28,29]. Depending on the tissue of origin, the isolated mammalian carboxylesterases display a wide variety of polymorphisms and therefore numerous specificities concerning substrates or inhibitors, some of them even show stereoselectivity [30]. In the rat, four types of carboxylesterases are known, designated hydrolase A, B, C and S. It has been suggested that even gender specific differences in enzyme activities exist [31,32].

Carboxylesterase enzymes are involved in the non-oxidative metabolism of alcohols via fatty acid conjugation. The influence of the lipase activity of carboxylesterases, where fatty acids are generated by the lipolysis of various triglycerides, is of special importance in the pathophysiology of alcohol-induced diseases: fatty acid-ethyl esters, generated from ethanol and fatty acids by the also known hydrolase-activity of carboxylesterases, can accumulate in various organs where they exert their toxicity by causing myocardial infarction, neurotoxicity, pancreatitis or even carcinomas [29].

Another important feature of carboxylesterase activities in connection with drug abuse is its role in cocaine-detoxification [33]. In the liver *N*-methylated toxic cocaine metabolites are inactivated via the formation of benzoylecgonine, which exerts no psychomotor stimulating activity anymore. In the presence of ethanol, a widespread form of co-abuse, changes the conditions for the inactivation of cocaine: in a transesterfication reaction, carboxylesterases catalyse the biosynthesis of the active metabolite cocaethylene [34]. This molecule causes the same behavioural effects as cocaine itself but is much more toxic, has stronger analgesic properties as well as a longer elimination half-life period [35]. It has been postulated that the preference of the ethanol-induced transesterfication in contrast to the normal detoxification pathway of cocaine (via hydrolysis) is caused by a hydrophobic ethanol-binding domain near the active center of the enzyme. This site is saturable and can be inhibited by non-reactive compounds such as acetone or dioxane [36]. In addition to alcohols, amines could also bind to this site because of their nucleophilicity [37]. Norharman is an amine by itself and as a molecule possessing a

tricyclic aromatic ring system it is a strong nucleophilic compound. Therefore, it has a great potential to influence the hydrolysis–transesterfication-mechanism. It remains to be elucidated whether it can act as a substrate or influences the enzyme activity by binding to the postulated hydrophobic domain near the active center of the enzyme [36]. Using a carboxylesterase inhibitor as possible competitor, preliminary radioligand binding experiments showed no changes of [^{3}H]norharman binding to solubilized rat liver binding sites (Greube, work in progress), suggesting that norharman does not bind to the active center of the enzyme. Kinetic experiments have to clarify whether norharman can modulate the enzyme activity.

The second norharman binding protein isolated in the present study, GRP78, is a member of the family of glucose-regulated proteins (GRPs), a subfamily of the group of heat-shock proteins (HSPs). They represent highly conserved proteins and are found in prokaryotes as well as in eukaryotes and plants. GRPs are often named ''chaperones'' or ''stress proteins'' as well, which has more in common with their physiological functions. They are not only inducible by cell-stress factors, but many of them are also essential cell proteins, necessary for cell viability [38–40]. GRP78 facilitates the refolding of misfolded proteins to regain the correct structure and it promotes their secretion through the membrane of the endoplasmic reticulum [41,42].

Concerning norharman and alcoholism, the most interesting feature of GRP78 is that it belongs (like some chaperones, various enzymes, receptors, or signal transduction molecules [43,44]) to the group of so-called ''ethanol responsive genes''. The induction by ethanol occurs at the transcriptional level and the mechanism seems to be different compared to ''classical'' inducers like brefeldin A, thapsigargine, tunicamycin, and A 23187; it even was reported that induction may split into two different mechanisms [45].

A second topic, which might be of interest, is the observation that a growing number of human diseases are discussed in connection with alterations of chaperone expression, including Alzheimer or Parkinson's disease and certain tumors [46–49]. Elevated norharman levels have also been detected in cerebrospinal fluid and blood plasma of patients with Morbus Parkinson [11]. The structural similarity of β-carbolines to the neurotoxin MPTP and its active metabolite MPP^+ suggests, similar to the cocaine detoxification pathway, a marker function of norharman for functionally disturbed detoxifying enzymes. A first set of binding experiments with [^{3}H]norharman and purified GRP78 seemed to point to an inhibitory role of the amine towards the protein, but these experiments using a purchased purified preparation of GRP78 gave no clear results. This may be, as we think, caused by the recombinant production of the protein, which makes it questionable if the GRP78 was in its natural, glycosylated conformation.

In order to take a closer look at the interactions between carboxylesterase enzymes, as well as GRP78 and norharman, and to get a better insight into their influence according drug dependence and co-mutagenicity, we may have reached a new starting point: in this study, the isolation of the ''norharman-related'' forms of these proteins via a newly synthesised affinity matrix has been described. These special protein preparations seem to be necessary for inhibition and induction studies which could be important in understanding their role in etiology of chronic alcohol abuse and neurodegenerative diseases. Especially regarding malfunction on protein activities in the presence of norharman and/or modified substrate specificities, it is necessary to scale up the purification protocol in order to get larger, specially defined protein preparations. In further studies, esterases and chaperones from various target organs can be purified to determine their functional and structural interrelationship to β-carbolines.

6. Nomenclature

BC	β-carboline
BCA	bicinchoninic acid
B_{max}	maximum number of binding sites
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]propane-1-sulfonic acid
d	diameter
dpm	decays per min
GRP	glucose-regulated protein

GTP	guanosine triphosphate
[^{3}H]	tritium
HSP	heat-shock protein
IC_{50}	displacer concentration at half-maximal displacement
IR	infrared
K_D	affinity constant
l	length
MOPS	3-*N*-morpholino-propanesulfonic acid
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine
NMR	nuclear magnetic resonance
P_2	crude cell membrane fraction
PAGE	polyacrylamide gel electrophoresis
Pd/C	palladium/carbon
PVDF	polyvinyl difluoride
SDS	sodium-dodecylsulphate
TEMED	tetramethylenediamine
TLC	thin layer chromatography
TRIS	2-amino-2-hydroxymethylpropane-1,3-diol

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