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INCREASED BINDING OF DESMETHYLIMIPRAMINE IN PLASMA OF PHENOBARBITAL-TREATED RATS

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Abstract—In plasma of untreated male Wistar rats the mean free fraction of desmethylimipramine (DMI) amounted to 10.5%. A five-day oral treatment with phenobarbital (PB) reduced it to 6.2%. A similar effect was produced in female Wistar rats and in male, but not in female Sprague–Dawley rats. Pretreatment with DDT did not alter DMI binding. The PB effect could not be attributed to the presence of PB or its metabolites in plasma nor to lower levels of endogenous compounds attached to plasma proteins. Studies on single DMI-binding proteins in plasma of male Wistar rats disclosed unchanged concentrations and binding properties of albumin and total lipoproteins following PB administration, while α_1-acid glycoprotein isolated from plasma of PB-treated animals bound DMI stronger than that from controls and contained a higher percentage of *N*-acetylneuraminic acid. The enhanced binding to a chemically altered α_1-acid glycoprotein species is at least one factor responsible for decreased tissue–plasma concentration ratios of DMI in PB-treated rats and constitutes an unusual type of drug interaction.

Tricyclic antidepressant drugs are basic amphiphilic compounds that strongly bind to plasma proteins but even more so to parenchymatous organs such that high tissue–plasma concentration ratios result. In an investigation of the effect of phenobarbital (PB) pretreatment on the kinetics of the antidepressant desmethylimipramine (DMI) in rats, an unusual type of interaction has become apparent. PB led to a decrease of brain–plasma and partly also of kidney–plasma and liver–plasma concentration ratios irrespective of the presence of high PB concentrations [1]. The possibility that this phenomenon was caused by an alteration of DMI plasma binding had to be taken into consideration. A direct interaction at protein binding sites was improbable because the PB effect persisted when the substance had largely been eliminated.

Plasma proteins known to bind tricyclic antidepressant drugs are albumin [2, 3], lipoproteins [4–7] and α_1-acid glycoprotein (α_1-AGP) [8, 9]. The concentrations of these proteins were determined in PB-pretreated and control rats and DMI binding to plasma and isolated proteins was studied in order to elucidate the mechanism of the pharmacokinetic interaction.

MATERIALS AND METHODS

Drugs. Tritium-labeled DMI (sp. act. 1 Ci/mmole), imipramine (IP, 2 Ci/mmole), and amitriptyline (2.1 Ci/mmole) were prepared as described previously [5]. Unlabeled DMI and IP were kindly donated by Ciba-Geigy (Basle, Switzerland).

Treatment of animals. Male and female rats of the Wistar and Sprague–Dawley strains were purchased from Ivanovas (Kisslegg, FRG) and fed a standard laboratory chow and tap water *ad lib.* When used for the experiments, males weighed 250–380 g. Food was removed 20 hr before sacrifice which was always done between 2 and 4 p.m.

The rats were pretreated with PB by giving 50 mg/kg in 5 ml/kg of water twice daily by gavage for 5 days; controls received water instead. The animals were mostly sacrificed 52 hr after the last PB dose. In some experiments one dose of 50 mg/kg was given by gavage followed by 1 g/l PB in the drinking water for 5 days right up to the time of death. In an experiment on the acute effect of PB administration 50 mg/kg was given 30 and 6 hr prior to death. Chlorophenothane (DDT, 100 mg/kg) was injected intraperitoneally in olive oil (2 ml/kg) and animals were killed after 6 days.

Under light ether anesthesia blood was drawn from the inferior caval vein into a heparinized syringe. Plasma was separated by centrifugation at 1000 *g* for 15 min and either stored at −20° or used immediately for the isolation of lipoproteins. Plasma PB levels were measured by thin-layer chromatography [10].

Isolation of proteins. Total lipoproteins were separated from plasma by ultracentrifugation [11], dialyzed in Visking cellulose tubing for 24 hr against 4 changes of 10 l water and concentrated using polyethyleneglycol (type 40000, Serva, Heidelberg, West Germany). For binding experiments they were diluted to the cholesterol concentration originally present in plasma. This required the addition of 8–10 vol. of 66 mM phosphate buffer pH 7.4 in 50 mM NaCl. The dilution of the buffer with about 10% of water did not affect the results. Albumin was isolated by gel filtration followed by anion-exchange chromatography [12] and did not show any contamination upon electrophoresis. The isolation of α_1-AGP [13] from 30–50 ml batches of plasma also resulted in a pure product.

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The purity of albumin and α_1-AGP was checked by electrophoresis in 13% polyacrylamide/0.1% sodium dodecylsulfate (SDS) gel at pH 8.3 in 25 mM Tris–200 mM glycine buffer containing 0.1% SDS. Since the staining procedure with Coomassie Brilliant Blue G-250 was less sensitive to α_1-AGP than to albumin, an admixture of 10% α_1-AGP to albumin or of 1% albumin to α_1-AGP would have been detected by electrophoresis.

Measurement of proteins. Total plasma protein and albumin were determined by the biuret and bromocresol green methods [14], respectively, using bovine serum albumin as standard. Plasma glycoproteins were precipitated as the "seromucoid" fraction [15]. Protein was measured by the biuret method and carbohydrates as detailed below. The concentrations of solutions of pure α_1-AGP were controlled by spectrophotometry using as reference value an extinction $E^{1\%}_{280}$ of 6.7 [16]. The protein content of these solutions was found to be 58% of total glycoprotein by the Lowry procedure [17], while Nagashima *et al.* [16] determined 51–58% on the basis of amino acid analyses.

Lipid analyses. Total cholesterol and triglycerides were measured using commercial kits (Boehringer, Mannheim, West Germany). Phospholipid-P was determined by mixing 0.05 ml of plasma with 1 ml of 1.2 N trichloroacetic acid and heating the precipitate with 0.5 ml of 70% perchloric acid to 170° until colourless. Phosphate was estimated by colorimetry [18] using KH_2PO_4 in perchloric acid as a standard. Plasma free fatty acids were determined by colorimetry [19].

Carbohydrate analyses. N-Acetylneuraminic acid was assayed according to Jourdian *et al.* [20]. Hexosamine analysis [21] was performed after hydrolysis in 3 M HCl for 4 hr at 100° [15]. In assays of neutral hexoses [22] a 1:1 mixture of D-galactose and D-mannose served as a standard.

Binding measurements. Free fractions of ^{3}H-labeled drugs in plasma and in protein solutions were determined by equilibrium dialysis for 6 hr under pH control [6]. DMI, IP, or amitriptyline were added to the buffer at concentrations of 50–63 ng/ml except in experiments designed for deriving DMI binding parameters. These were determined graphically from Scatchard plots [23] according to Klotz and Hunston [24]. All binding experiments were carried out in duplicate, the coefficient of variation being 5.0%. The dialysis time of 6 hr proved sufficient to obtain equilibrium not only with plasma [6] but also with lipoproteins isolated from human plasma.

PB was added to plasma as a solution in phosphate buffer and the mixture was left at room temperature for 30 min prior to dialysis. Palmitic acid was dissolved in ethanol, the solution spread on the bottom of a tube and the solvent evaporated under a stream of nitrogen. Plasma was added and the acid dissolved by gentle shaking and storing overnight at 4°.

Charcoal treatment of plasma was carried out by the method of Chen [25] as modified by Craig *et al.* [26] using Norit A (Serva, Heidelberg, West Germany). Control samples were acidified to pH 3.0 and after 1 hr adjusted back to pH 7.4, but no charcoal was added.

Binding to erythrocytes was either measured in fresh blood or in washed cells. To blood samples of 200 µl, [^{3}H]DMI was added to a concentration of 138 ng/ml. After incubation at 37° for 15 min and centrifugation 50 µl of plasma was removed for scintillation counting. An incubation time of 15 min had been shown to be sufficient for attaining binding equilibrium. The cell volume was derived from blood hemoglobin according to

$$\text{hematocrit (\%)} = 3.6 \cdot \text{hemoglobin (g/100 ml)}$$ [27].

Washed red cells were obtained by suspending the packed cells twice in five volumes of isotonic phosphate buffer pH 7.4 and centrifuging. A suspension of 1 ml of washed cells in 5 ml of buffer was incubated after addition of 280 ng [^{3}H]DMI for 15 min at 37°. Following centrifugation ^{3}H was measured in 0.2 ml of supernatant.

Binding calculations. In order to obtain an estimate of the DMI fraction bound to single proteins, the equilibrium according to the law of mass action

$$\frac{[\text{DMI}_{\text{free}}] \cdot n[\text{P}_{\text{free}}]}{[\text{DMI}_{\text{bound}}]} = K_d$$

(With K_d = dissociation constant, [P] = concentration of protein with n binding sites) was transformed into

$$\frac{[\text{DMI}_{\text{free}}] \cdot (n[\text{P}] - [\text{DMI}_{\text{bound}}])}{[\text{DMI}_{\text{bound}}]} = K_d$$

$$\frac{[\text{DMI}_{\text{free}}]}{[\text{DMI}_{\text{bound}}]} \cdot n[\text{P}] = K_d + [\text{DMI}_{\text{free}}]$$

$$\frac{[\text{DMI}_{\text{bound}}]}{[\text{DMI}_{\text{free}}]} = \frac{n[\text{P}]}{K_d + [\text{DMI}_{\text{free}}]}$$

When $K_d \gg [\text{DMI}_{\text{free}}]$, the equation simplifies to

$$[\text{DMI}_{\text{bound}}]/[\text{DMI}_{\text{free}}] = n[\text{P}] \cdot K_a$$

(K_a = association constant) and, in the presence of m binding proteins, to

$$[\text{DMI}_{\text{bound}}]/[\text{DMI}_{\text{free}}] = \sum_{i=1}^{i=m} n_i[\text{P}_i] \cdot K_{ai}$$

Patient serum. Blood was obtained from 16 epileptics, most of them outpatients, who were under long-term treatment with phenobarbital or primidone. Three patients each received in addition phenytoin and/or carbamazepine and one each mephobarbital or valproate, respectively. Serum was stored frozen and binding of ^{3}H-labeled amitriptyline was measured in duplicate.

RESULTS

Drug binding to plasma

In Wistar rats PB pretreatment led to a significantly enhanced binding of DMI to plasma. The effect was equally great whether the PB administration was carried out until the time of sacrifice or whether it was terminated 52 hr earlier (Table 1). The influence of PB was also present when rats received 25 mg/kg of DMI i.p. 5 hr before sacrifice (data not shown). Single 50 mg doses of PB given 30 and 6 hr before exsanguination and DDT pretreatment did not alter DMI binding to plasma of

Table 1. Free fraction of DMI and PB concentration in plasma of unpretreated and PB-treated rats

Strain	Sex	Control group free DMI (%)	PB group free DMI (%)	plasma PB (μg/ml)
PB treatment till to sacrifice				
Wistar	male	10.0 ± 0.8 (3)	6.5 ± 0.8** (5)	32.5 ± 15.4
PB treatment terminated 52 hr before sacrifice				
Wistar	male	10.5 ± 1.3 (12)	6.2 ± 0.9** (12)	2.4 ± 0.8
	female	11.3 ± 1.2 (5)	9.2 ± 0.6* (5)	
Sprague-Dawley	male	11.3 ± 0.6 (6)	8.2 ± 1.4** (12)	3.6 ± 0.9
	female	12.2 ± 1.1 (10)	11.6 ± 1.5 (10)	

Values are means ± S.D. (n).
Significant differences from the control group by two-tailed t test are *$P < 0.01$ or **$P < 0.001$.

male Wistar rats. The free fraction of IP in plasma of untreated male Wistar rats amounted to 15.2 ± 2.1% (mean ± S.D., $n = 5$), while 52 hr after the last dose of a 5 days' PB treatment it was reduced to 8.7 ± 1.6% ($n = 6$, $P < 0.001$).

The effect of continued PB administration was present in Wistar rats of both sexes, though in females it was smaller than in males. In contrast, it could not be detected in female Sprague–Dawley rats, whereas males did react (Table 1).

All further experiments were carried out in male Wistar rats given the last PB dose 52 hr before sacrifice.

Modified Scatchard plots for DMI binding to plasma revealed at least two sets of binding sites (Fig. 1). The association constant of the first set was slightly but significantly ($P < 0.05$) higher in pretreated ($1 \cdot 10^6\,M^{-1}$) than in control rats ($0.85 \cdot 10^6\,M^{-1}$). This resulted from a comparison of the regression coefficients of the linear parts of the plots fitted by a least squares method. Also the capacity of the first set defined by the maximal DMI concentration bound to this set in plasma was increased after pretreatment ($1.5 \cdot 10^{-5}$ vs $1 \cdot 10^{-5}$ M). Due to experimental scatter, a valid comparison between groups of the association constants (around $10^4\,M^{-1}$) and capacities (around 10^{-4} M) of the second set could not be carried out. The binding differences at low DMI levels must have been due predominantly to the differences in the first set.

PB added at concentrations measured in pretreated rats did not appreciably influence DMI binding to control plasma. Palmitic acid led to a slight increase of unbound DMI (from 6.4 to 7.1%) when 1.5 moles/mole albumin was added to plasma. Charcoal treatment reduced the free DMI fraction to 76% the original value in plasma from pretreated rats and to 71% in that from controls (Fig. 2).

In four duplicate experiments, plasma from PB-pretreated rats was dialysed against control plasma after [^{3}H]DMI addition to both half-cells. Tritium counts per ml of the former plasma exceeded those in the latter by a factor of 1.50 ± 0.08 (S.D.).

DMI binding to erythrocytes

Under the conditions used, washed erythrocytes from PB-pretreated and control rats bound 72.8 ± 2.5% and 72.6 ± 4.9% (mean ± S.D., $n = 14$), respectively. In contrast, when DMI was incubated in whole blood, significantly less (56.5 ± 4.0%) was bound to red cells from PB-treated rats than to those from controls (66.9 ± 3.9%, $n = 14$, $P < 0.001$). Since the hemoglobin concentration was very similar in the two groups (14.2 ± 0.5 vs 14.7 ± 0.5 g/100 ml), the difference could be ascribed to the varying extent of plasma protein binding.

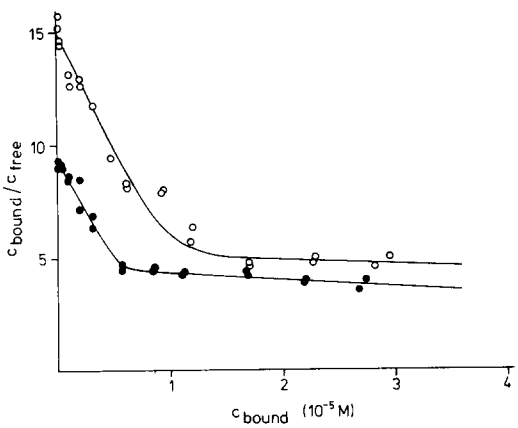

Fig. 1. Modified Scatchard plots for the binding of DMI to plasma of unpretreated (●——●) and PB-treated (○——○) male Wistar rats.

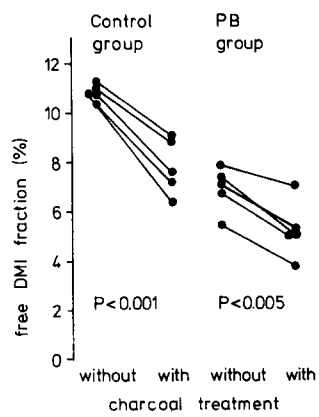

Fig. 2. Influence of charcoal treatment on the binding of DMI to plasma of unpretreated and PB-treated male Wistar rats. Statistical evaluation by paired t-test (two-tailed).

Table 2. Plasma proteins and protein components in unpretreated and PB-treated rats (last dose 52 hr before sacrifice)

Compound	Control group	PB group
Total protein (mg/ml)	62.9 ± 4.6 (20)	65.1 ± 3.3 (26)
Albumin (mg/ml)	28.3 ± 1.5 (24)	28.9 ± 1.6 (30)
Glycoproteins (protein content, mg/ml)	2.25 ± 0.28 (6)	2.55 ± 0.40 (6)
Free fatty acids (μmol/ml)	1.21 ± 0.25 (14)	1.01 ± 0.26 (9)
Cholesterol (mg/100 ml)	59.8 ± 15.4 (11)	70.6 ± 18.8 (11)
Triglycerides (mg/100 ml)	49.0 ± 18.0 (10)	36.9 ± 6.4 (10)
Phospholipid-P (mg/100 ml)	3.38 ± 0.68 (8)	3.97 ± 1.10 (8)

Values are means ± S.D. (*n*).

Plasma protein analyses

Concentrations of total plasma protein and of proteins known to bind tricyclic psychoactive drugs were determined using the parameters listed in Table 2. None of these proved to change significantly as a consequence of PB treatment. Electrophoresis of glycoproteins isolated by differential precipitation [15] showed that the procedure worked less well with rat than with human plasma. Whereas the preparation from the latter contained only albumin and a protein of mol. wt 24,000 as admixtures to α_1-AGP, the precipitate from rat plasma was separated into at least 5 bands the strongest of them corresponding to α_1-AGP purified by chromatography [13]. Carbohydrate analyses of the precipitate did not reveal significant differences between the two groups of rats.

DMI binding to isolated proteins

Total lipoproteins prepared from plasma of PB-treated rats bound DMI to the same extent as those prepared from control plasma (free fraction 34.2 vs 32.2%, total cholesterol 51.3 vs 50.6 mg/100 ml). Similarly, albumin at a concentration of 30 mg/ml left 28.0 and 29.0%, respectively, of DMI unbound in experiments with and without PB administration.

In contrast, α_1-AGP prepared from plasma of PB-treated rats bound DMI more than the corresponding fraction from control plasma. Its *N*-acetylneuraminic acid content was slightly but significantly increased (Table 3), while the electrophoretic mobility was unchanged. The *N*-acetylneuraminic acid concentration in plasma was, however, nearly identical in the two groups. It amounted to 570 ± 50 (S.D.) μg/ml in 9 controls and to 591 ± 31 μg/ml in 9 PB-treated rats.

Table 3. DMI binding to and carbohydrate content of α_1-AGP purified from plasma of control and PB-treated rats. Binding was measured at an α_1-AGP concentration of 0.45 mg/ml corresponding to a protein concentration of 0.26 mg/ml

	Control group	PB group
Free DMI (%)	68.0 ± 8.6 (5)	48.7 ± 8.1** (4)
Carbohydrates (%)		
Neutral hexoses	14.6; 17.3; 23.8	13.8; 15.2; 15.4
N-Acetylglucosamine	10.2	7.4; 9.7
N-Acetylneuraminic acid	2.9 ± 0.1 (4)	3.4 ± 0.2* (4)

Values are means ± S.D. (*n*) unless results from single experiments are given separately.
*$P < 0.02$; **$P < 0.01$.

Amitriptyline binding to serum of PB-treated patients

Serum of patients in whom a seizure disorder was controlled by phenobarbital, partly in conjunction with other anticonvulsants, bound amitriptyline to the same extent (free fraction 8.2 ± S.D. 1.2%, $n = 16$) as did plasma of healthy volunteers (free fraction 7.6 ± 1.0%, $n = 26$).

DISCUSSION

The unbound fraction of DMI in plasma from male Wistar rats (10.5 ± 1.3%) was slightly less than that in normal human plasma (15.3 ± 1.6%) [6]. Lower values were observed by Borgå *et al.* [28] in both species, probably due to pH shifts during ultrafiltration.

In the present experiments it could be demonstrated that a schedule of PB treatment that produced decreased tissue–plasma distribution coefficients of DMI in rats [1] led to enhanced binding of DMI to plasma. This enhancement was great enough to explain the decrease of brain–plasma concentration ratios to an average of 65% of control values 5 hr after DMI injection. Other tissue–plasma concentration ratios were decreased less consistently pointing to the possibility that binding to tissues (liver, lung, kidney) may also have been affected. In contrast, binding to erythrocytes remained unchanged.

The PB effect was not due to the presence of PB or of its metabolites in plasma. This resulted from its absence upon PB addition *in vitro* and at short intervals after single doses given *in vivo*, from its persistence in rats treated for 5 days to a time when the PB concentration had declined to a very low level, and from binding differences when ultrafiltrable substances were allowed to exchange between plasma from pretreated and control rats. Furthermore, the altered binding was not a direct consequence of an induction of drug-metabolizing enzymes, since DDT proved to be inactive.

Charcoal treatment of plasma which removes free fatty acids [25] and a number of additional compounds [26] improved DMI binding in the PB-treated and in the control group to the same extent. This effect which contrasts with the failure of the same procedure to enhance propranolol binding to normal or uremic rabbit plasma [29] must have been due to the adsorption of endogenous binding inhibitors. Their concentrations were obviously not changed by PB treatment of rats, and their chemical nature remained obscure. Fatty acids cannot be considered as candidates, since palmitic acid only minimally reduced DMI binding to rat plasma and at higher concentrations enhanced the binding of IP to human albumin [30].

Thus, an alteration of one or more of the DMI-binding proteins seemed to underlie the PB effect. The proteins studied in this respect were those known to bind tricyclic psychoactive drugs in human plasma (see Introduction). Albumin and total lipoproteins isolated from rat plasma proved to bind DMI appreciably, but PB treatment of rats did not change the fraction bound. In the case of lipoproteins, this applied in spite of a nonsignificant increase of plasma cholesterol and phospholipid levels which may correspond to the HDL cholesterol increase produced

by PB in male Wistar rats [31]. That albumin levels are not affected by PB treatment of rats has also been observed by Yacobi *et al.* [32, 33]; consequently, these authors did not detect changes in the serum binding of acidic drugs and bilirubin. A slight contamination of isolated albumin with α_1-AGP could not be excluded by electrophoretic analyses, but it was unlikely in view of the isolation procedure that included anion-exchange chromatography.

PB treatment did, however, lead to an increased binding of DMI to isolated α_1-AGP that was accompanied by an alteration of its carbohydrate composition, namely an increase of *N*-acetylneuraminic acid. The altered α_1-AGP did not resemble the precursor form of the protein in liver which was found not to contain *N*-acetylneuraminic acid [16]. Reports in the literature [13, 16, 34, 35] on carbohydrate contents of α_1-AGP prepared from rats of different strains deviate considerably from one another. The value of 2.9% *N*-acetylneuraminic acid in α_1-AGP from control plasma is lower than data from previous studies, but in none of these has the same strain been investigated.

Mean plasma or serum concentrations of α_1-AGP as measured by immunoelectrophoresis in Donryu, Wistar King A [35] and Buffalo rats [16] amounted to 0.77, 0.54 and 0.17 mg/ml, respectively. Therefore an intermediate concentration of 0.45 mg/ml was used in experiments with the purified protein. However, the observed difference in DMI binding to the preparations from control and PB-treated rats can serve to explain the binding difference in plasma only if one assumes that here the α_1-AGP level is appreciably higher. Two lines of evidence speak in favor of this assumption. First, a free DMI fraction of 10.5% in plasma of control rats and of 6.2% in that of PB-pretreated animals cannot be accounted for by the binding values measured on the three isolated proteins. In analogy with the binding kinetics of other tricyclic antidepressants [9], one can assume that at the ligand concentration used, the free DMI level is small compared to the dissociation constants such that the simplified formula (see Materials and Methods) is applicable. Taking into account the free fractions observed with isolated albumin and lipoproteins, isolated α_1-AGP from control rats at the concentration present in plasma should leave 20% of DMI unbound and that from PB-treated rats 8.6%. This reasoning is, of course, only valid unless further plasma proteins contribute to DMI binding or binding is affected by interactions of plasma constituents.

Second, α_1-AGP constituted the major component of the "seromucoid" fraction with a protein content of 2.25 mg/ml plasma in control rats. About 30% of human plasma "orosomucoid" ($=\alpha_1$-AGP) was found to escape detection in this fraction due to coprecipitation with other proteins [15]. It is not known to which extent such a coprecipitation occurs in rat plasma. Possibly a chemically altered α_1-AGP as it was present in PB-treated rats coprecipitates to a larger extent such that a quantitative change in addition to the qualitative one may have eluded detection. Thus, it seems possible that the enhanced DMI plasma binding following PB treatment of rats was a consequence of the presence of a chemically altered α_1-AGP. Alternatively, the treatment may have changed the binding properties of an additional plasma protein the potential of which to bind tricyclic psychoactive drugs has not been described hitherto. The PB effect was dependent on the strain and sex of rats and no indication of a similar phenomenon was obtained in man.

The inconsistent or lacking effect of the PB pretreatment on lung–plasma and kidney–plasma concentration ratios of DMI [1] is probably due to the fact that α_1-AGP can be taken up into peripheral tissues [36]. The presence of newly synthesized α_1-AGP in liver will lead to enhanced drug binding and can serve to explain not only the largely unchanged liver–plasma concentration ratio of DMI, but perhaps also the considerably reduced lung–liver, kidney–liver and brain–liver ratios of its metabolite didesmethylimipramine in PB-treated rats [1]. Possibly the same mechanism underlies the PB-induced decrease of kidney–liver and brain–liver concentration ratios of metabolites of the phenothiazine drug perazine in rats [37].

In conclusion, PB pretreatment of rats may lead to an altered distribution of basic amphiphilic compounds in the organism which can at least partly be ascribed to the presence of an altered α_1-AGP in plasma and possibly also in liver.

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