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Short communication

[^{3}H]GBR 12935 binding in platelets from poor and extensive cytochrome *P*-4502D6 metabolizers

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Abstract

Previous studies have indicated that part of the binding of [^{3}H] {1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl) piperazine dihydrochloride} ([^{3}H]GBR 12935) to human platelets is to a piperazine acceptor site, which might be associated with cytochrome *P*-450IID6 (CYP4502D6, debrisoquine-4-hydroxylase). Due to mutant *CYP4502D6* alleles, 5–10% of Caucasians are poor metabolizers of CYP4502D6 substrates such as debrisoquine and dextromethorphan. In the present study, possible differences in binding characteristics of [^{3}H]GBR 12935 in platelets from CYP4502D6 poor and extensive metabolizers were investigated. The most prominent finding was a gender difference, with males having significantly higher K_d values than females. There were no differences in B_{max}. After correction for gender, there was a tendency towards higher K_d values in poor metabolizers than in extensive metabolizers, although the difference was not statistically significant. Whether this finding corresponds to reduced CYP4502D6 activity is a matter of further investigation.

Keywords: Cytochrome *P*-4502D6; Extensive metabolizer; [^{3}H]GBR 12935 binding; Platelet, human; Piperazine acceptor site; Poor metabolizer

1. Introduction

The radioligand [^{3}H] {1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl) piperazine dihydrochloride} ([^{3}H]GBR 12935) labels the neuronal dopamine uptake site and functions as a marker for dopaminergic neuron terminals in brain. [^{3}H]GBR 12935 also recognizes a 'piperazine acceptor site' in brain. The designation implies that this site is recognized by piperazine derivatives such as flupenthixol and piflutixol (Andersen, 1987). Also in human platelet membranes, the binding of [^{3}H]GBR 12935 has yielded characteristics indicating that part of the binding is to a piperazine acceptor site (Gordon et al., 1994).

The molecular identity and functional significance of the piperazine acceptor site have not yet been established. Some data indicate that there might be an association with the cytochrome *P*-450 (CYP450) system; in canine brain the piperazine acceptor site has been identified as CYP4502D1, which corresponds to human CYP4502D6 (Niznik et al., 1990; Tyndale et al., 1991), and in human brain an association between the piperazine acceptor site and CYP4502D6 has been demonstrated (Allard et al., 1994). In human platelets, the pharmacological characteristics of [^{3}H]GBR 12935 binding were recently investigated. Part of the binding was inhibited by compounds known as inhibitors of [^{3}H]GBR 12935 binding to CYP4502D6 in liver and brain, suggesting that this binding fraction in platelets is associated with CYP4502D6 (Norlén and Allard, 1997).

In humans, CYP4502D6 (debrisoquine-4-hydroxylase) has been demonstrated mainly in liver. The enzyme is responsible for the metabolism of more than 40 different drugs, such as antidepressants, neuroleptics and antiarrhythmics. The *CYP4502D6* gene on human chromosome 22 is highly polymorphic. At least 20 different alleles have been described (Stüven et al., 1996), some of them harbouring mutations leading to the poor metabolizer phenotype. This autosomal recessive metabolic deficiency affects 5–10% of Caucasians and may lead to adverse reactions upon administration of drugs in standard doses. According to our previous study (Norlén and Allard, 1997),

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part of the [^{3}H]GBR 12935 binding in platelets might be to CYP4502D6. Therefore, possible differences in binding characteristics in platelets from CYP4502D6 poor and extensive metabolizers respectively, were investigated in the present study.

2. Materials and methods

2.1. Subjects

Thirty-four subjects took part in the investigation, which was approved by the Ethics Committee at the University of Umeå. All subjects were healthy, as assessed by medical history, physical examination and routine blood chemistry tests. They were recruited from a population of more than 500 volunteers phenotyped with respect to the CYP4502D6 polymorphism by means of the test drug dextromethorphan. The 34 subjects included 16 (11 men and 5 women) poor metabolizers of dextromethorphan and 18 (10 men and 8 women) extensive metabolizers of dextromethorphan. The mean age of the extensive metabolizer group was 38 ± 2 years (mean $\pm$ S.E.M.) (range 23–51 years) and of the poor metabolizer group 29 ± 2 years (range 23–43 years) ($P = 0.006$). When dividing the subjects with respect to gender, the mean age of the men was 30 ± 2 years (mean $\pm$ S.E.M.) (range 23–50 years) and of the women 40 ± 3 years (range 23–51 years) ($P = 0.002$). The body weight was 70 ± 3 kg (mean $\pm$ S.E.M.) in the extensive metabolizer group and 71 ± 3 kg in the poor metabolizer group. As expected, the mean body weight was significantly higher in males than in females (77 ± 2 and 60 ± 2 kg, respectively) ($P = 0.0001$). All individuals had been entirely drug-free for at least 2 weeks.

2.2. CYP4502D6 phenotyping

After intake of 50 mg dextromethorphan hydrobromide (Tussidyl mixture, 2 mg/ml, TIKA, Lund, Sweden), urine was collected for 10 h. Dextromethorphan and its *O*-demethylated metabolite dextrorphan were analyzed by a capillary gas chromatography method published earlier (Spigset et al., 1997). Dextromethorphan/dextrorphan ratios > 0.90 defined the CYP4502D6 poor metabolizers, whereas ratios < 0.90 defined the CYP4502D6 extensive metabolizers. The poor metabolizers had metabolic ratios between 0.92 and 77.11, whereas the extensive metabolizers had metabolic ratios between 0.02 and 0.86.

2.3. Tissue preparation

A total of 75 ml whole blood was collected from each individual. The samples were centrifuged at $180 \times g$ for 15 min at 20°C. Assay buffer (50 mM Tris · HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) was then added 1:1 to the supernatant, which was then centrifuged at $3000 \times g$ for 20 min at 4°C. The pellets were washed with 20 ml of assay buffer and then recentrifuged at $3000 \times g$ for 10 min at 4°C. The pellets were homogenized with $10 + 10$ ml of assay buffer using a Kinematica Polytron homogenizer (Luzern, Switzerland), at a setting of 6, for 7 s. The pellets were centrifuged at $15\,000 \times g$ for 10 min at 4°C and frozen at -70°C until assay.

On the day of experiment, the tissue was washed in $10 + 10$ ml assay buffer and centrifuged at $18\,000 \times g$ for 10 min. The pellets were finally homogenized at a setting of 4, for 10 s, and then resuspended in the assay buffer to a mean final protein concentration of 470 μg protein/ml (range 270–710 μg protein/ml).

2.4. Compounds

[^{3}H]GBR 12935 (40–45.7 Ci/mmol) was purchased from New England Nuclear, Boston, MA, USA. *cis*-(*Z*)-Flupenthixol was obtained as a gift from H. Lundbeck, Copenhagen, Denmark.

2.5. [^{3}H]GBR 12935 binding

The binding of [^{3}H]GBR 12935 was carried out for 60 min at 25°C in a total volume of 2.0 ml. The incubation volume contained 250 μl of tissue homogenate, 250 μl of radioligand, 250 μl of displacing drug or buffer and 1250 μl assay buffer (50 mM Tris · HCl, 120 mM NaCl, 0.01% bovine serum albumin, pH 7.4). The concentrations of [^{3}H]GBR 12935 ranged from 0.25 to 30 nM. The experiments were carried out in duplicate.

After the addition of 6 ml of ice-cold assay buffer, the homogenates were filtered through Whatman GF/C filters using a 24-channel cell harvester (Brandel, Gaithersburg, MD, USA) Finally, the filters were washed with three 6-ml rinses of the buffer and the radioactivity determined by liquid scintillation spectroscopy.

Specific [^{3}H]GBR 12935 binding to the piperazine acceptor site was defined as the difference between [^{3}H]GBR 12935 binding in the absence and presence of 0.3 μM *cis*-flupenthixol (Norlén and Allard, 1997).

Protein determination was performed according to Markwell et al. (1978).

2.6. Data analysis and statistics

B_{max} and K_d values were determined by least squares linear regression analysis of Scatchard plots derived from the saturation experiments. Student's *t*-test and covariate analysis were used for comparisons between groups. The criterion for statistical significance was $P < 0.05$.

3. Results

B_{max} was 680 ± 74 fmol/mg protein (mean $\pm$ S.E.M.) in the poor metabolizer group and 648 ± 78 fmol/mg protein in the extensive metabolizer group ($P = 0.772$). K_d

was 5.77 ± 0.81 nM in the poor metabolizer group and 4.29 ± 0.52 nM in the extensive metabolizer group (Fig. 1A), indicating a lower binding affinity in the poor metabolizer group. The difference was, however, not statistically significant ($P = 0.126$). Representative Scatchard plots, one from each group, are depicted in Fig. 2.

There were no correlations between age and the B_{max} or K_d values ($r = -0.19$ and -0.28, respectively). Neither were there any correlations between body weight and the B_{max} or K_d values ($r = 0.25$ and 0.31, respectively).

When analyzing possible gender differences in binding parameters, no significant differences in density of binding sites were found; in male subjects (poor metabolizers plus extensive metabolizers), B_{max} was 725 ± 78 fmol/mg protein and in female subjects (poor metabolizers plus extensive metabolizers), B_{max} was 564 ± 49 fmol/mg protein ($P = 0.144$). In contrast, significant differences in binding affinity were found; K_d for male subjects was 5.73 ± 0.66 nM, and for female subjects 3.78 ± 0.56 nM ($P = 0.048$) (Fig. 1B).

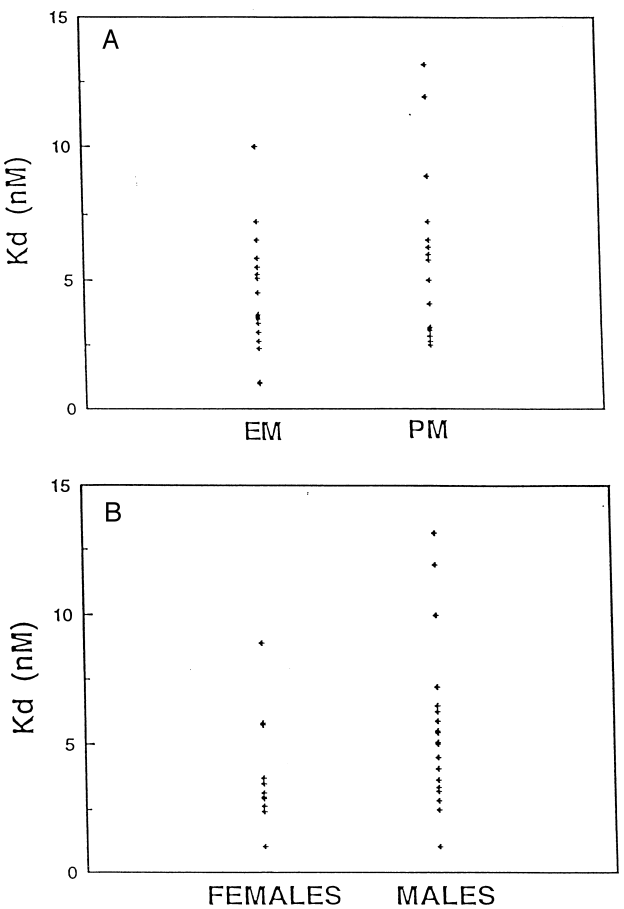

Fig. 1. Distribution of K_d values obtained from least-squares linear regression analysis of Scatchard plots from saturation studies on [^{3}H]GBR 12935 binding to piperazine acceptor sites in platelets. (A) Data from CYP4502D6 poor and extensive metabolizers. K_d was 4.29 ± 0.52 nM (mean $\pm$ S.E.M.) in the extensive metabolizer group and 5.77 ± 0.81 nM in the poor metabolizer group. The difference in K_d between the groups was not statistically significant ($P = 0.126$). (B) Data from the same subjects as in (A) divided into males and females. K_d was 5.73 ± 0.66 nM (mean $\pm$ S.E.M.) in males and 3.78 ± 0.56 nM in females. There was a statistically significant difference in K_d between the two groups ($P = 0.048$).

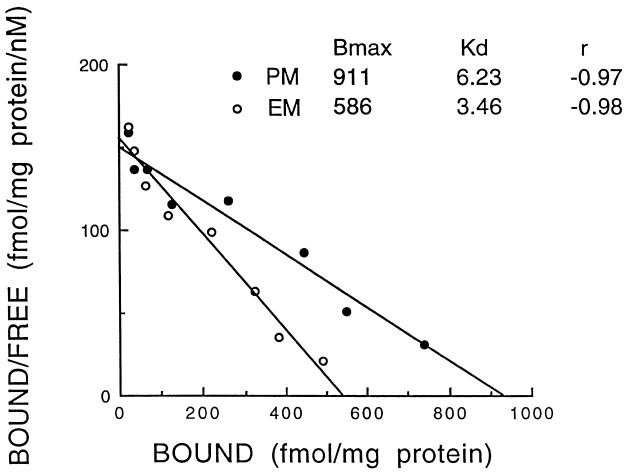

Fig. 2. Representative Scatchard plots of [^{3}H]GBR 12935 binding to piperazine acceptor sites in platelet preparations from one poor metabolizer and one extensive metabolizer, respectively. The [^{3}H]GBR 12935 concentrations ranged from 0.25 to 30 nM. The values for B_{max} (fmol/mg protein), K_d and the correlation coefficient r are indicated.

After correction for gender using covariance analysis, the differences in K_d between the poor metabolizers and the extensive metabolizers remained grossly unchanged ($P = 0.19$).

There was no correlation between the concentrations of tissue protein and the K_d values ($r = -0.28$).

4. Discussion

The principal finding in this study was that males had significantly lower binding affinity for [^{3}H]GBR 12935 than females. This discrepancy seems not to be related to differences in CYP4502D6 activity since the metabolism of debrisoquine is not affected by gender (Hietanen et al., 1991; Bock et al., 1994). The observed gender difference could imply that the binding is influenced in a competitive manner by hormonal factors not yet identified, or that the properties of the piperazine acceptor ligand binding sites are differently developed secondary to unknown gender-related factors.

No significant differences in binding parameters were obtained between the poor metabolizer and the extensive metabolizer groups, although higher K_d values were found in the poor metabolizer group, a tendency that persisted after correction for gender. Although the difference was not statistically significant, some possibilities favouring that [^{3}H]GBR 12935 binding could be associated with CYP4502D6 can be stated. The reduced binding affinity in poor metabolizers could in fact indicate impairments in enzymatic function among the poor metabolizers or, alternatively, that CYP4502D6 is not completely expressed in

platelets. Further possibilities that could be accounted for might be that [^{3}H]GBR 12935 binds to CYP450 isoenzymes other than CYP4502D6, several of which are gender-specific (Fletcher et al., 1994; Gleiter and Gundert-Remy, 1996), or that mutant *CYP4502D6* alleles in poor metabolizers might produce enzymes not metabolically active but still harbouring piperazine acceptor sites.

A shortcoming to the study might be the age differences with respect to gender and metabolic capacity; women being significantly older than men, and the extensive metabolizers being significantly older than the poor metabolizers. Age was, however, not significantly correlated to either the K_d or the B_{max} values and, consequently, the age differences appear not to influence the binding parameters.

The function and significance of the piperazine acceptor site are incompletely known. The present study revealed, as a conclusion, that there was a significant gender difference in the affinity of piperazine acceptor sites for the binding of [^{3}H]GBR 12935. There was also a tendency towards reduced affinity for [^{3}H]GBR 12935 binding to piperazine acceptor sites among poor metabolizers compared with extensive metabolizers, although the difference was not statistically significant. The association between the piperazine acceptor site and CYP4502D6 in platelets has not been convincingly demonstrated. If such a connection exists, it should be taken into account that gender-related factors probably exert an important influence upon that link.

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