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SHORT COMMUNICATION

Specific Binding of 1-[2-(Diphenylmethoxy)ethyl]-4-(3-phenyl propyl) piperazine (GBR-12935), an Inhibitor of the Dopamine Transporter, to Human CYP2D6

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ABSTRACT. The binding of [^{3}H]1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl propyl) piperazine (GBR-12935), an antagonist of the dopamine transporter, to human P450s expressed in yeast cells was investigated. Among the ten forms of human P450 tested (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, and 3A4), [^{3}H]GBR-12935 bound most strongly to CYP2D6. The calculated K_d of [^{3}H]GBR-12935 binding to CYP2D6 was 42.2 nM, indicating that GBR-12935 has a high affinity for CYP2D6. The characteristics of [^{3}H]GBR-12935 binding to CYP2D6 were investigated by competitive studies using several chemicals. The binding of [^{3}H]GBR-12935 to CYP2D6 was not changed by dopamine, suggesting that these binding sites are not dopamine-sensitive binding sites. The binding of [^{3}H]GBR-12935 to CYP2D6 was decreased partially by substrates or inhibitors of CYP2D isoforms (quinine, quinidine, propranolol, bufuralol, imipramine, and desipramine). By means of binding studies using several forms of expressed human P450, we demonstrated that the CYP2D isoform is one GBR-12935 binding site that is insensitive to dopamine. BIOCHEM PHARMACOL 53;12:1937–1939, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cytochrome P450 2D forms; human; dopamine transporter; piperazine acceptor

CYP2D§ isoforms have been found not only in the liver but also in extrahepatic organs, such as the brain, kidney, adrenal glands, and lymphocytes, by means of catalytic studies, immunohistochemical examinations, and RT-PCR [1–5]. However, the physiological and pharmacological functions of CYP2D isoforms in these extrahepatic organs have not been clarified. The presence of multiple [^{3}H]GBR-12935 binding sites in rat and human brains has been demonstrated [6, 7]. One of these is a dopamine transporter. Another is insensitive to dopamine and is called a "piperazine acceptor site." The function of the piperazine acceptor site remains unknown. Niznik *et al.* [8] investigated the characteristics of the piperazine acceptor by competitive studies using various compounds and other studies using antibodies. They concluded that the piperazine acceptor site is present not only in the brain but also in the liver, and that this site is the CYP2D isoform. However, binding assays using isolated forms of P450 have not been investigated to date. Liu *et al.* [9] demonstrated the existence of MBP in several organs, including the brain. We clarified that MBP has properties similar to those of CYP2D1 [10]. The K_d value of [^{3}H]mepyramine binding to MBP is close to that of CYP2D1. Quinidine and quinine inhibit mepyramine binding to both MBP and CYP2D1. Further, specific antibodies against CYP2D1 recognize MBP on western blotting. Our results indicate that neither competitive nor inhibitive binding studies using specific compounds, nor immunoadsorbent study using antibodies, can distinguish between CYP2D1 and MBP. Studies of CYP2D isoforms must focus on the existence of CYP2D-like proteins (e.g. MBP). In this study, we assayed [^{3}H]GBR-12935 binding activity using ten forms of human P450 expressed in yeast cells and investigated the kinetics or characteristics of [^{3}H]GBR-12935 binding to expressed CYP2D6.

MATERIALS AND METHODS

Chemicals

[^{3}H]GBR-12935 (45.7 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA, U.S.A.). Propranolol was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dopamine and GBR-12935 were obtained from Research Biochemicals International (Natick, MA, U.S.A.). Bufuralol was obtained from the

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§ *Abbreviations:* CYP, cytochrome P450; GBR-12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl propyl) piperazine; GBR-12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenyl-2-propyl) piperazine; P450, cytochrome P450; RT-PCR, reverse transcriptase-polymerase chain reaction; and MBP, mepyramine binding protein.

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Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Expression of Human P450 *in* Saccharomyces, Cerevisiae

Ten forms (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, and 3A4) of human P450 were individually expressed in *S. cerevisiae*. A detailed description of PCR-amplification, the expression of human P450 cDNA in yeast cells, and its properties has been reported previously [11].

GBR-12935 Binding Assay

P450 (3 pmol) was incubated with [^{3}H]GBR-12935 (10 nM) at 25° for 60 min in a final volume of 250 μL of 0.05 M sodium phosphate buffer, pH 7.5. Nonspecific binding was defined in the presence of 10 μM unlabeled GBR-12935. In the case of the competitive binding assay, several chemicals (10 μM) as competing ligands were added in the reaction mixture. Saturation studies were performed with six concentrations (10–100 nM) of [^{3}H]GBR-12935. Then the reaction mixture was applied to a gel filtration column (9.0 × 28.0 mm) containing Sephadex G-25 (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 0.05 M sodium phosphate buffer, pH 7.5. The radioactivity levels of the fractions containing proteins were measured in a Pico-Fluor™40 scintillation mixture (Beckman Instruments, Inc., Fullerton, CA, U.S.A.).

RESULTS AND DISCUSSION

To clarify whether GBR-12935 directly binds to P450, we examined [^{3}H]GBR-12935 binding to ten forms of human P450 expressed in the microsomal fraction from yeast cells at a constant concentration (10 nM) of [^{3}H]GBR-12935 (Fig. 1). Among the ten forms of human P450, [^{3}H]GBR-12935 had the highest binding activity to CYP2D6. We found that [^{3}H]GBR-12935 also bound to CYP2A6, CYP2C18, and CYP2E1, but these binding levels were less than half of the binding level to CYP2D6. Binding to the other P450s was very low. Specific [^{3}H]GBR-12935 binding to CYP2D6 was determined at six concentrations of [^{3}H]GBR-12935, ranging from 10 to 100 nM. The binding of GBR-12935 to CYP2D6 was analyzed by Scatchard plots, and the dissociation constant (K_d), the number of receptors (B_{max}), and the Hill coefficient were calculated. The Scatchard plots of the GBR-12935 saturation curves were linear. The Hill coefficient was 0.93, indicating that GBR-12935 binds to one or more sites of CYP2D6 with the same apparent affinity. The calculated K_d value of GBR-12935 to CYP2D6 was 42.2 nM, and the B_{max} was 10.9 pmol/mg of protein. The K_d value of GBR-12935 binding to human dopamine transporter transfected in COS-7 cells is 1.08 nM

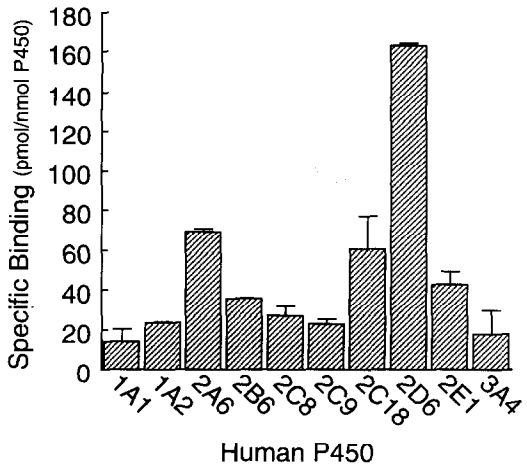

FIG. 1. GBR-12935 binding activity to human P450s expressed in yeast. The reaction mixture, containing 3 pmol human P450 and 10 nM [^{3}H]GBR-12935 in a final volume of 0.25 mL of 0.05 M sodium phosphate buffer, pH 7.5, was incubated in duplicate for 60 min at 25° in the absence or presence of 10 μM unlabeled GBR-12935. Data represent the means ± SD of triplicate experiments.

[12]. The affinity of GBR-12935 for CYP2D6 was weaker than that for the dopamine transporter.

We studied the competitive effects of several chemicals upon GBR-12935 binding to CYP2D6 (Fig. 2). Total GBR-12935 binding to CYP2D6 almost disappeared when unlabeled GBR-12935 was used. This indicates that most of the GBR-12935 binding to CYP2D6 is specific binding. Total GBR-12935 binding to CYP2D6 was not changed by dopamine, indicating that CYP2D6 has a dopamine-insensitive GBR-12935 binding site. Total GBR-12935 binding

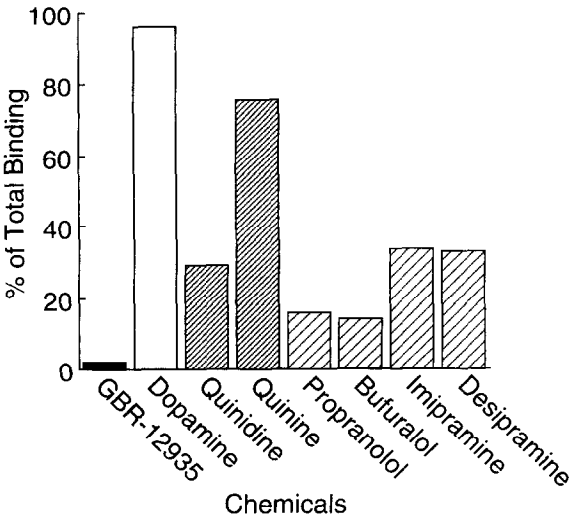

FIG. 2. Effects of several chemicals on GBR-12935 binding to CYP2D6. The reaction mixture, containing 3 pmol CYP2D6 and 10 nM [^{3}H]GBR-12935 in a final volume of 0.25 mL of 0.05 M sodium phosphate buffer, pH 7.5, was incubated for 60 min at 25° in the absence (total binding) or presence of a 10 μM concentration of various chemicals as a competing ligand. Total binding to CYP2D6 was 156.8 pmol/nmol P450. Data represent the means of duplicate experiments.

was reduced to 29.3 and 75.6% by quinidine and quinine, respectively, which are specific and potent inhibitors of CYP2D enzymatic activities [13]. The binding to CYP2D6 was decreased markedly by propranolol, bufuralol, imipramine, and desipramine, which are typical substrates for CYP2D isoforms.

Microsomal fractions from a human lymphoblastoid cell line expressing CYP2D6 oxidize GBR-12909, an aryldialkylpiperazine [14]. This study showed that CYP2D6 binds to compounds such as piperazines and plays a role in metabolizing those compounds in the liver. In the brain, the GBR-12935 binding site in the cerebral cortex or prefrontal cortex is a piperazine acceptor site, which has low affinity for mazindol [15, 16]. In the rat central nervous system, there are high levels of CYP2D1-like immunoreactivity and CYP2D mRNA signal in the cerebral cortex [17]. The distributions of the piperazine acceptor and CYP2D isoforms in the brain are similar. The reports cited above support the notion that the piperazine acceptor site in the liver and brain is a CYP2D isoform [1, 8, 18]. However, direct evidence that GBR-12935 binds to CYP2D has not been obtained. In this study, we directly assayed the GBR-12935 binding activity using several forms of human P450. Our results clarified that GBR-12935 binds to CYP2D isoforms with high affinity and that the CYP2D isoform is one of the GBR-12935 binding sites that are insensitive to dopamine.

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