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σ RECOGNITION SITES IN BRAIN AND PERIPHERAL TISSUES

CHARACTERIZATION AND EFFECTS OF CYTOCHROME P450 INHIBITORS

DIANE L. DEHAVEN-HUDKINS,* LORRAINE F. LANYON, FELICIA Y. FORD-RICE and MARK A. ATOR

Department of Enzymology and Receptor Biochemistry, Sterling Winthrop Pharmaceuticals Research Division, Collegeville, PA 19426–0900, U.S.A.

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Abstract—Binding to σ sites in subcellular fractions of brain and in crude homogenates from peripheral tissues of the guinea pig was characterized with the [^{3}H]ligands (+)pentazocine and di(2-tolyl)guanidine (DTG). The inhibitory effects of representative σ compounds and cytochrome P450 inhibitors were evaluated in guinea pig tissues, and the effects of cytochrome P450 induction on σ binding in the rat were investigated. For both ligands, the majority of sites were localized to the microsomal fractions. The K_D values for [^{3}H](+)pentazocine- or [^{3}H]DTG-labeled σ sites in guinea pig liver and testes were 2-fold lower than those in brain and heart. The number of sites labeled by [^{3}H](+)pentazocine varied, with an order of liver > testes > brain > heart. In contrast, the B_{max} values for [^{3}H]DTG-defined σ sites were greatest in testes, followed by liver, brain and heart. The rank order of potency for representative σ and P450 compounds was similar in brain, liver and testes for both [^{3}H]ligands, and the potency of selective compounds to displace σ binding in guinea pig liver failed to correlate with their abilities to inhibit cytochrome P450IID1 activity in human liver. Following induction of cytochrome P450IIB1 with phenobarbital or cytochrome P450IA1 with β-naphthoflavone, neither the affinity nor the number of σ sites was altered in rat brain or liver. These results suggest that σ sites in the periphery are similar to those in the brain, and that the σ binding site is not identical with cytochrome P450IIB1, P450IA1 or P450IID1.

Key words: σ receptor; cytochrome P450; enzyme induction; di(2-tolyl)guanidine; (+)pentazocine

σ Recognition sites have been identified in both brain and peripheral tissues of several species [see Refs. 1–3]. Ligands such as [^{3}H](+)SKF-10,047† [4–7], [^{3}H]dextromethorphan [8, 9], [^{3}H]haloperidol [5, 10, 11], [^{3}H]DTG [11–13], [^{3}H](+)3-PPP [10, 11, 13] and [^{3}H](+)pentazocine [13, 14] all bind to the σ site in liver and brain homogenates with high affinity, and σ sites have also been identified in kidney [9], testes, ovary [15], spleen [16] and adrenals [9, 15]. Moreover, the presence of multiple σ binding sites in brain and periphery has been proposed [13, 17, 18], where the σ_1 subtype is labeled by [^{3}H]-(+)pentazocine, and [^{3}H]DTG labels the σ_2 site.

The structure–activity relationships for compounds that bind to the σ site are quite broad [see Refs. 1 and 19–22], and the lack of a definitive functional event that correlates with σ binding potency [21, 23–26] suggests that the binding site for σ ligands may not represent a pharmacological receptor in the classical sense. McCann *et al.* [24] originally proposed that the σ site may be a membrane-bound enzyme based on the high concentration of binding sites for [^{3}H](+)SKF-10,047 in liver and its microsomal localization in brain. The broad structure–activity relationships for compounds interacting with the σ site may be indicative of an association with the microsomal oxygenase system [24, 27]. In rat, the P450 inhibitors proadifen (SKF-525A) and alaproclate potently inhibit the binding of several σ ligands, with IC_{50} values ranging from 9 to 84 nM in brain and approximately 10 nM in liver [28]. GBR-12909, a compound that inhibits cytochrome P450IID1 in canine brain [29, 30] also potently inhibits the binding of the σ ligands [^{3}H]-(+)pentazocine [14], [^{3}H]dextromethorphan [31], [^{3}H](+)3-PPP [31, 32] and [^{3}H]DTG [22, 31] in guinea pig brain, with affinities of less than 10 nM. Down-regulation of [^{3}H]DTG-labeled σ sites was observed in liver and brain following the administration of the steroid spironolactone, which depletes cytochrome P450 [33].Dwoskin and coworkers [34] reported that the number of σ sites labeled with [^{3}H](+)3-PPP was decreased by 27% in brain and 26% in liver of the Dark Agouti rat strain, which lacks the gene product for cytochrome P450IID1 [35].

* Corresponding author: Dr. Diane L. DeHaven-Hudkins, Department of Enzymology and Receptor Biochemistry, Sterling Winthrop Pharmaceuticals Research Division, P.O. Box 5000, Collegeville, PA 19426–0900. Tel. (215) 983-5650; FAX (215) 983-5293.

† Abbreviations: BMY-14802, α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine-butanol; DTG, di(2-tolyl)guanidine, GBR-12909, 1-[2-*bis*(4-fluorophenyl)-methoxy]ethyl]-4-[3-phenylpropyl]piperazine; 3-PPP, 3-(3-hydroxyphenyl)-*N*-propylpiperidine; and SKF-10,047, *N*-allylnormetazocine.

To better understand the proposed role of cytochrome P450 in the effects of σ compounds, we initially examined the binding affinity and density of the σ site in subcellular fractions of brain, and we compared σ binding in brain with binding in peripheral tissues that contain high levels of cytochrome P450: the liver, testes and heart [36]. The σ ligands [^{3}H](+)pentazocine and [^{3}H]DTG were used to determine the relative roles of proposed σ subtypes [17, 18] in the effects on the cytochrome P450 system. We also explored the possibility of a relationship between the cytochrome P450 system and the σ recognition site by determining if (1) the binding affinities of compounds in brain, liver and testes correlated with their affinities to inhibit the noninducible enzyme cytochrome P450IID1 [37], and (2) up-regulation of the σ site is observed following induction of cytochrome P450IIB1 and P450IA1 isozymes in brain and liver. These P450 subforms were chosen for study because of reports that all three are present in the brain of several species [29, 30, 38–42].

MATERIALS AND METHODS

σ Radioreceptor assays. Tissue was prepared from male Hartley guinea pigs (Hazelton Laboratories, Denver, PA) or male Sprague–Dawley rats (Taconic), which were anesthetized with CO_2 and killed by decapitation. All animal care and use procedures were in accord with the "Guide for the Care and Use of Laboratory Animals" (NIH Publ. No. 86–23, 1985) and the Animal Welfare Act, and were approved by the Sterling Winthrop Pharmaceuticals Research Division Institutional Animal Care and Use Committee. Assays for [^{3}H]-(+)pentazocine [14] and [^{3}H]DTG [22] were conducted as previously described.

Subcellular fractionation. Subcellular fractionation of guinea pig brain was performed by the method of Craviso and Musacchio [43]. Briefly, whole brain minus cerebellum was dissected and homogenized in 10 vol. of 0.32 M sucrose using a glass mortar and Teflon pestle. The homogenate was centrifuged at 900 *g* for 10 min at 4°, and the pellet was washed twice with 0.32 M sucrose and centrifuged as described above to yield the P1 nuclear fraction. The supernatants from the P1 fractions were combined and centrifuged at 11,500 *g* for 20 min at 4°, and the resulting crude P2 pellet was washed with 0.32 M sucrose and recentrifuged once. The supernatants from the crude P2 fraction were centrifuged at 100,000 *g* for 60 min at 4° to give the P3 microsomal fraction. The P2 pellet was osmotically shocked with 10 vol. of cold H_2O, and homogenized; then the homogenate was centrifuged at 11,500 *g* for 20 min at 4° to yield the final pelleted P2 mitochondrial fraction. The supernatant and buffy coat from the P2 fraction were centrifuged at 100,000 *g* for 60 min to yield the P4 synaptic membrane fraction.

Enzyme induction studies. Cytochromes P450IIB1 and P450IA1 were induced in male Sprague–Dawley rats by the intraperitoneal injection of sodium phenobarbital (75 mg/kg; P450IIB1) or a saline control, and β-naphthoflavone (40 mg/kg; P450IA1) or a corn oil control over a 4- or 3-day period, respectively [44]. Throughout the injection period, food and water were freely available. The animals were killed 24 hr after the last dose, followed by immediate removal of brain and liver. The brains and livers were homogenized, and the homogenate was divided into portions for binding assays or for microsomal preparation for the enzyme assay.

Cytochrome P450 activities were measured using the 7-ethoxycoumarin-*O*-deethylase assay, which detects a broad spectrum of P450 isozymes [44]. This assay was performed using the method of Naslund *et al.* [39] except that 10 mM glucose-6-phosphate and 1 U/mL glucose-6-phosphate dehydrogenase were employed as an NADPH-regenerating system, in place of isocitrate and isocitrate dehydrogenase. Time–course studies were run in brain and liver at enzyme concentrations of 0.25 and 0.5 mg/mL in uninduced, 0.125 and 0.25 mg/mL in phenobarbital-induced, and 0.0625 and 0.125 mg/mL in β-naphthoflavone-induced rats.

Data analysis. Equilibrium binding parameters and inhibition constants for the binding of test compounds were calculated using the EBDA/LIGAND program [45], purchased from Elsevier/Biosoft. Enzyme activities were calculated by linear fits of the data using Microsoft Excel (Microsoft Corp., Redmond, WA) and CricketGraph (Cricket Software, Malvern, PA). Correlation coefficients using linear regression analysis, and other statistical analyses were calculated using the PHARM/PCS program [46].

Chemicals. [^{3}H](+)Pentazocine was prepared by custom synthesis at New England Nuclear (Boston, MA) to a specific activity of 35–37 Ci/mmol.

Table 1. Subcellular distribution of σ binding sites in guinea pig brain

Fraction	[^{3}H](+)pentazocine		[^{3}H]DTG	
	K_D (nM)	B_{max} (fmol/mg protein)	K_D (nM)	B_{max} (fmol/mg protein)
P_1 nuclear	3.3 ± 0.4	2,007 ± 347	53 ± 2	3,579 ± 761
P_2 mitochondrial	4.1 ± 0.2	652 ± 77	49 ± 11	1,528 ± 425
P_3 microsomal	4.0 ± 0.2	3,562 ± 173	64 ± 12	6,244 ± 786
P_4 synaptic plasma membrane	3.8 ± 0.9	1,552 ± 16	46 ± 3	3,112 ± 288

Data are the means ± SEM of at least three separate determinations.

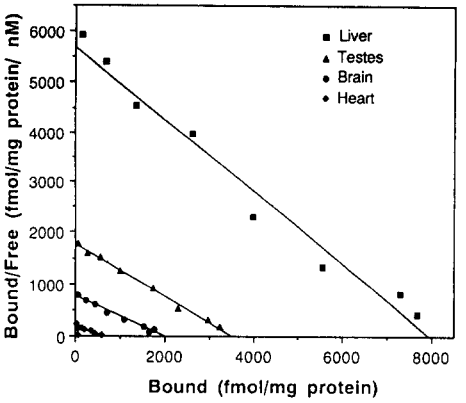

Fig. 1. Equilibrium binding analyses of σ sites labeled by [^{3}H(+)pentazocine in various guinea pig tissues. Data are taken from representative experiments. K_D values (in nM) were: heart, 3.1 ± 0.1; brain, 2.9 ± 0.6; testes, 1.9 ± 0.1; and liver, 1.4 ± 0.1. B_{max} values (in fmol/mg protein) were: heart, 889 ± 235; brain, 1,998 ± 97; testes, 3,594 ± 244; and liver, 8,068 ± 974. Values are means ± SEM, N = 3.

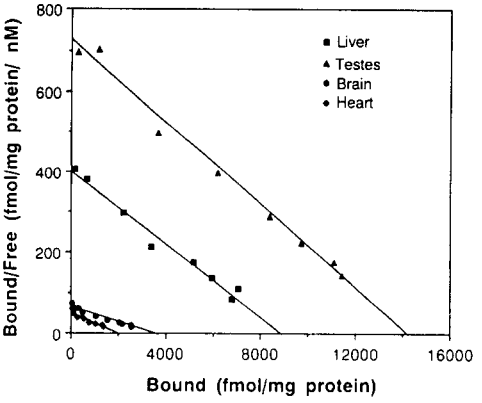

Fig. 2. Equilibrium binding analyses of σ sites labeled by [^{3}H](+)DTG in various guinea pig tissues. Data are taken from representative experiments. K_D values (in nM) were: heart, 62 ± 24; brain, 43 ± 3; liver, 20 ± 2; and testes, 20 ± 2. B_{max} values (in fmol/mg protein) were: heart, 2,198 ± 263; brain, 3,367 ± 162; liver, 10,083 ± 1,825; and testes, 13,391 ± 956. Values are means ± SEM, N = 3.

[^{3}H]DTG (30–60 Ci/mmol) was purchased from New England Nuclear. BMY-14802 was a gift from Bristol-Myers Squibb. All other compounds and reagents were purchased from the Sigma Chemical Co. (St. Louis, MO) or Research Biochemicals Inc. (Natick, MA).

RESULTS

In brain the binding of both σ ligands was greatest in the microsomal fraction (Table 1). σ Sites were also numerous in the nuclear and synaptic plasma membrane fractions, with the least number of sites present in the mitochondrial fraction. The affinities for the respective ligands were similar, but the number of sites labeled by [^{3}H]DTG was approximately 2-fold greater than that by [^{3}H]-(+)pentazocine in all fractions.

Both [^{3}H](+)pentazocine and [^{3}H]DTG bound to a single site in all four tissues examined (Figs. 1 and 2). The K_D values for σ sites labeled by both [^{3}H](+)pentazocine and [^{3}H]DTG in liver and testes

Table 2. Inhibition constants of representative σ and cytochrome P450 compounds at [^{3}H](+)pentazocine sites in guinea pig tissues

Compound	Brain		Liver		Testes	
	K_i (nM)	nH	K_i (nM)	nH	K_i (nM)	nH
Haloperidol	0.6 ± 0.1	0.89 ± 0.03	1.7 ± 0.3	1.05 ± 0.05	0.4 ± 0.1	0.87 ± 0.07
(+)Pentazocine	2.1 ± 0.1	0.91 ± 0.01	3.0 ± 0.3	0.99 ± 0.05	1.7 ± 0.1	0.91 ± 0.01
GBR-12909	22 ± 3	1.02 ± 0.14	118 ± 18	1.29 ± 0.10	134 ± 0.3	1.33 ± 0.10
Proadifen	30 ± 7	1.22 ± 0.05	59 ± 11	0.96 ± 0.03	25 ± 4	0.83 ± 0.03
l-Lobeline	53 ± 10	0.97 ± 0.06	90 ± 15	0.99 ± 0.01	35 ± 3	0.87 ± 0.03
(+)3-PPP	80 ± 12	0.94 ± 0.02	121 ± 13	0.97 ± 0.02	63 ± 2	0.98 ± 0.05
DTG	107 ± 21	1.00 ± 0.02	101 ± 16	0.96 ± 0.01	54 ± 1	0.92 ± 0.02
(−)Pentazocine	112 ± 13	0.94 ± 0.03	151 ± 28	1.02 ± 0.03	84 ± 15	0.99 ± 0.04
(+)SKF-10,047	182 ± 25	0.93 ± 0.01	298 ± 59	0.96 ± 0.03	120 ± 13	1.03 ± 0.05
BMY-14802	265 ± 46	0.95 ± 0.02	290 ± 45	0.92 ± 0.03	122 ± 17	0.87 ± 0.02
Chlorpromazine	418 ± 75	0.98 ± 0.05	1,407 ± 172	1.34 ± 0.06	635 ± 31	1.15 ± 0.06
(−)3-PPP	737 ± 146	0.96 ± 0.02	855 ± 80	0.96 ± 0.01	513 ± 87	0.98 ± 0.05
Rimcazole	836 ± 45	1.01 ± 0.05	1,604 ± 301	1.16 ± 0.11	731 ± 43	1.03 ± 0.02
Amitryptyline	1,616 ± 11	1.01 ± 0.05	260 ± 47	1.04 ± 0.01	112 ± 8.4	0.99 ± 0.03
(−)SKF-10,047	4,728 ± 1,097	1.04 ± 0.03	7,266 ± 988	1.05 ± 0.02	3,790 ± 72	0.83 ± 0.03
(−)Sparteine	8,189 ± 914	1.00 ± 0.07	13,662 ± 1,906	0.98 ± 0.01	7,844 ± 509	0.96 ± 0.02
Quinidine	16,602 ± 4,707	1.37 ± 0.18	27,935 ± 2,448	1.25 ± 0.03	18,483 ± 1,218	1.15 ± 0.06

Data are the means ± SEM of at least three experiments performed in triplicate. nH = Hill coefficient. Final concentration of [^{3}H](+)pentazocine = 0.4 nM.

Table 3. Inhibition constants of representative σ and cytochrome P450 compounds at [^{3}H]DTG sites in guinea pig tissues

Compound	Brain		Liver		Testes	
	K_i (nM)	nH	K_i (nM)	nH	K_i (nM)	nH
Haloperidol	5 ± 0.4	0.65 ± 0.01	1.8 ± 0.2	0.86 ± 0.04	3.1 ± 0.2	0.95 ± 0.04
GBR-12909	5 ± 1	0.47 ± 0.04	49 ± 10	0.87 ± 0.14	4 ± 1	0.64 ± 0.05
l-Lobeline	36 ± 9	0.90 ± 0.13	13 ± 1	0.80 ± 0.01	29 ± 3	0.92 ± 0.03
DTG	63 ± 10	0.71 ± 0.04	22 ± 1	0.97 ± 0.02	22 ± 2	1.05 ± 0.03
Amitryptyline	133 ± 23	0.99 ± 0.13	136 ± 9.9	0.91 ± 0.01	320 ± 17	0.97 ± 0.04
BMY-14802	149 ± 38	0.57 ± 0.04	39 ± 6.1	0.85 ± 0.02	29 ± 3.8	0.87 ± 0.01
(−)Pentazocine	176 ± 23	0.74 ± 0.02	97 ± 11	0.83 ± 0.05	112 ± 2	0.88 ± 0.01
(+)3-PPP	240 ± 39	0.67 ± 0.06	65 ± 6	0.82 ± 0.02	141 ± 12	0.89 ± 0.02
Rimcazole	254 ± 17	0.70 ± 0.06	504 ± 27	0.96 ± 0.06	162 ± 18	0.91 ± 0.05
Proadifen	304 ± 16	1.05 ± 0.06	309 ± 11	0.97 ± 0.03	572 ± 44	1.11 ± 0.10
Chlorpromazine	371 ± 39	0.82 ± 0.09	487 ± 39	1.12 ± 0.03	558 ± 64	1.29 ± 0.03
(+)Pentazocine	513 ± 150	0.72 ± 0.05	190 ± 10	0.78 ± 0.02	558 ± 66	1.04 ± 0.07
(−)3-PPP	1,016 ± 148	0.74 ± 0.02	320 ± 14	0.91 ± 0.03	563 ± 14	0.95 ± 0.01
Quinidine	1,896 ± 410	0.55 ± 0.08	3,050 ± 204	0.63 ± 0.02	83 ± 20	0.53 ± 0.01
(−)Sparteine	1,997 ± 388	0.73 ± 0.14	2,495 ± 386	0.84 ± 0.01	950 ± 102	0.79 ± 0.02
(−)SKF-10,047	7,200 ± 267	0.91 ± 0.03	7,720 ± 893	1.12 ± 0.05	9,673 ± 491	1.11 ± 0.03
(+)SKF-10,047	7,845 ± 1,015	0.72 ± 0.03	4,488 ± 945	0.85 ± 0.03	15,694 ± 727	1.11 ± 0.03

Data are the means ± SEM of at least three experiments performed in triplicate. nH = Hill coefficient. Final concentration of [^{3}H]DTG = 4 nM.

were approximately 2-fold higher than those in brain and heart. The greatest number of sites labeled by [^{3}H](+)pentazocine was found in liver, followed by testes, brain and heart. In contrast, the B_{max} for [^{3}H]DTG-labeled sites was highest in testes, followed by liver, brain and heart. [^{3}H]DTG labeled approximately twice the number of sites as did [^{3}H]-(+)pentazocine in heart and brain, and four times as many sites in testes, whereas the number of sites labeled by each ligand in liver was approximately equal.

The rank order of potency for various compounds to displace [^{3}H](+)pentazocine binding was generally similar in brain, liver and testes (Table 2). The only notable exceptions to this were for GBR-12909, which was approximately 5-fold more potent in brain than in the peripheral tissues, and amitryptyline, which was 6-fold more potent in liver and 14-fold more potent in testes than in brain. The cytochrome P450 inhibitors *l*-lobeline and proadifen were potent inhibitors of binding in each tissue examined, with K_i values of 25–90 nM. The cytochrome P450IID1 substrate sparteine and the inhibitor quinidine were weak to inactive, with K_i values of 7.8 to 27.9 μM.

The rank order of potency at the σ site labeled by [^{3}H]DTG was also similar in all tissues (Table 3). Certain compounds such as (+)pentazocine, (+)3-PPP, (−)3-PPP, BMY-14802, DTG, haloperidol, and *l*-lobeline were 3- to 5-fold more potent in liver than in brain. BMY-14802, DTG and quinidine were 3- to 22-fold more potent in testes than in brain. However, in spite of these minor differences in potency, the rank order of potency for [^{3}H]DTG binding in all three tissues strongly correlated (r = 0.78–0.96, $P < 0.01$), as did that of [^{3}H]-(+)pentazocine (r = 0.99–1.0, $P < 0.01$).

A correlation of binding affinity for either [^{3}H]-(+)pentazocine- or [^{3}H]DTG-labeled σ sites in guinea pig liver versus the K_i for human liver sparteine monooxygenase [47] failed to establish a relationship between cytochrome P450IID1 inhibition and affinity at the σ site (r = −0.06 for [^{3}H]-(+)pentazocine, 0.07 for [^{3}H]DTG; Table 4).

Administration of phenobarbital or β-naphthoflavone caused a robust induction of 3-fold for cytochrome P450IIB1 and 4-fold for cytochrome P450IA1, respectively, in rat liver, but enzyme activity in brain could not be detected for either P450 isoform (Table 5). The numbers of sites labeled by [^{3}H](+)pentazocine were not increased significantly following enzyme induction in either brain or liver. In brain, the B_{max} values (in fmol/mg protein) for each treatment group were: saline, 376 ± 9; phenobarbital, 426 ± 19; corn oil, 509 ± 24; β-naphthoflavone, 606 ± 87. In liver, the values were: saline, 5586 ± 186; phenobarbital, 6264 ± 353; corn oil, 6279 ± 345; β-naphthoflavone, 6095 ± 358 fmol/mg protein. Although induction of cytochrome P450IIB1 in liver resulted in a 21% increase in [^{3}H]DTG-labeled σ sites, and induction of cytochrome P450IA1 caused a 22% increase in the number of [^{3}H]DTG-labeled σ sites, these differences did not reach statistical significance (Table 5). B_{max} values for [^{3}H]DTG following enzyme induction were unchanged in brain. The K_D values were similar in vehicle- or drug-treated rats for both ligands in either brain or liver.

DISCUSSION

Our observation of a preferential microsomal distribution in guinea pig brain for both [^{3}H]-(+)pentazocine- and [^{3}H]DTG-labeled σ recognition sites is consistent with previous observations using a variety of ligands to label σ sites in subcellular fractions of brain from several species

Table 4. Comparison of affinities at σ recognition sites in guinea pig liver versus inhibition of cytochrome P450IID1 in human liver

Compound	Guinea pig liver, K_i (nM)		Human liver, K_i (μM)
	[^{3}H](+)pentazocine	[^{3}H]DTG	Sparteine monooxygenase*
Haloperidol	1.7	1.8	1
Proadifen	59	309	5
l-Lobeline	90	13	0.1
GBR-12909	118	49	0.1
Amitryptyline	260	136	50
Chlorpromazine	1407	487	7
Sparteine	13662	2495	30
Quinidine	27935	3050	0.06

* Taken from [29] and [47].

Table 5. [^{3}H]DTG equilibrium binding parameters and enzyme induction values following cytochrome P450IIB1 induction with phenobarbital and cytochrome P450IA1 induction with β-naphthoflavone in rat tissues

Treatment	Tissue	K_D (nM)	B_{max} (fmol/mg protein)	Specific activity (nmol/min/mg protein)
Saline	Brain	50.2 ± 1.8	1,954 ± 41	ND*
Phenobarbital	Brain	55.4 ± 1.8	2,279 ± 223	ND
Corn oil	Brain	54.1 ± 2.0	2,332 ± 176	ND
β-Naphthoflavone	Brain	57.2 ± 4.9	2,611 ± 364	ND
Saline	Liver	23.2 ± 2.1	20,627 ± 2,230	1.71 ± 0.10
Phenobarbital	Liver	21.9 ± 1.7	24,919 ± 2,757	5.86 ± 0.33†
Corn oil	Liver	22.9 ± 0.8	21,241 ± 1,534	1.62 ± 0.11
β-Naphthoflavone	Liver	24.1 ± 1.4	25,945 ± 2,307	6.71 ± 1.49†

Values are the means ± SEM of four separate determinations performed in triplicate.
* ND = not detected.
† Significantly different from control (one-tailed *t*-test): $P < 0.01$.

[1, 24, 28, 43, 48, 49]. The possible identity of the σ site as a cytochrome P450 isoform was originally hypothesized based on the localization of [^{3}H]-(+)SKF-10,047 binding to microsomal fractions of rat brain [24]. A subsequent detailed analysis of the subcellular distribution of haloperidol-sensitive sites found an enrichment of σ sites in both the microsomal and myelin fractions, with low amounts in mitochondrial and nuclear fractions [48]. In contrast, binding to σ sites labeled by [^{3}H]dextromethorphan in guinea pig was enriched in the synaptic plasma membrane fraction, followed by nuclear, mitochondrial and microsomal fractions, and Rosenthal analysis of the binding to each fraction revealed that fractions other than microsomes contained low-affinity binding sites, while the microsomal fraction contained high-affinity sites for [^{3}H]dextromethorphan [43]. Subfractionation of [^{3}H]dextromethorphan-labeled σ sites in microsomes revealed that the distribution of these sites was similar to the distribution of NADPH–cytochrome *c* reductase, a marker of endoplasmic reticulum [43]. [^{3}H](+)SKF-10,047 binding was also colocalized with NADPH–cytochrome *c* reductase, but the enrichment of σ binding sites in myelin suggested that σ sites are not localized exclusively to endoplasmic reticulum [48]. Itzhak *et al.* [49] performed a direct comparison of the subcellular distribution of sites labeled by the σ ligands [^{3}H]-(+)SKF-10,047, [^{3}H](+)3-PPP and [^{3}H]DTG in mouse brain. Although all three ligands preferentially bound to the microsomal fraction, [^{3}H](+)SKF-10,047 and [^{3}H](+)3-PPP also demonstrated significant binding to the mitochondrial fraction. Differences between the subcellular localization of σ sites as defined by various ligands may be due to the affinities of certain σ ligands for other receptors or may be due to methodological differences between the studies, but the general observation is that the majority of σ sites are found in the microsomal fraction of brain.

However, the microsomal localization of the σ site is not necessarily indicative of an enzymatic function. Ligands for dopaminergic [50], muscarinic [51], opioid [43] and β-adrenergic [52] receptors label high amounts of binding in microsomal fractions of brain. Cross-contamination of fractions is often a problem in these types of studies, and microsomal localization of binding sites may be indicative of a receptor that undergoes rapid synthesis or transport

[53]. Further, in brain, cytochrome P450 is localized to non-synaptic mitochondria compared to microsomes in a ratio of 3:1 in both rat and guinea pig [54]. The low amounts of σ binding to mitochondria argue against the identity of the σ site in brain as cytochrome P450.

The tissue distribution of σ binding sites labeled by [^{3}H](+)pentazocine and [^{3}H]DTG in guinea pig was similar to results in previously published reports using other σ ligands, although subtle differences were observed in the binding of these ligands. [^{3}H]-DTG labeled four times as many sites in testes as did [^{3}H](+)pentazocine, while the number of sites labeled by each ligand in liver was approximately equal, and [^{3}H]DTG labeled twice as many sites as did [^{3}H](+)pentazocine in brain and heart (Figs. 1 and 2). Further, the affinities of both ligands in liver and testes were approximately 2-fold greater than in brain and heart (Figs. 1 and 2). Large numbers of σ sites labeled by [^{3}H](+)SKF-10,047, [^{3}H]-(+)pentazocine or [^{3}H]DTG are present in rat liver [7, 13]. Musacchio *et al.* [9] observed high levels of [^{3}H]dextromethorphan binding to liver and brain of guinea pig, with low amounts in heart, and Wolfe *et al.* [15] detected large numbers of σ sites in testis using [^{3}H]haloperidol as the ligand.

We were unable to demonstrate any evidence for the hypothesis that a specific σ subtype was related to cytochrome P450. Binding to the σ_1 site predominates in brain, whereas σ_2 binding predominates in liver [13]. However, the rank order of potency for σ reference compounds and compounds acting at cytochrome P450 in liver, testis and brain was similar for [^{3}H](+)pentazocine, which labels the σ_1 site, and [^{3}H]DTG, which labels the σ_2 site [13, 18]. Based on our B_{max} values in these two tissues, our ratio of 4:1 for liver:brain binding of [^{3}H](+)pentazocine and 3:1 for [^{3}H]DTG is similar to that of Hellewell *et al.* [13]. The lack of effect of caramiphen and carbetapentane, proposed σ_1 ligands [18, 55] on [^{3}H]dextromethorphan binding in liver and kidney [9], suggests subtle differences in central versus peripheral σ binding sites. Our data examining various σ and P450 reference compounds failed to find any remarkable differences in affinities for [^{3}H]-(+)pentazocine-labeled σ sites in brain, liver or testes (Table 2), suggesting that the sites labeled by [^{3}H](+)pentazocine in the brain and periphery are pharmacologically similar, as demonstrated by the high correlation ($r > 0.99$) between K_i values in each of the tissues examined. The situation is less clear with [^{3}H]DTG, where Rosenthal analysis revealed binding to a single site in brain (Fig. 2 and [22]), but the slopes for certain σ reference compounds were less than one, indicating binding to more than one site. In contrast, the slopes for binding in liver and testes almost uniformly gave slopes approximately equal to one, with the exception of shallow displacement curves for GBR-12909 in testes and quinidine in liver and testes (Table 3). The data suggest that [^{3}H]DTG binds to a second site in guinea pig brain that is either absent or present in very low amounts in the periphery. However, in spite of differences in potency for certain compounds, a significant correlation still exists between K_i values in the three tissues ($r \geq 0.78$). Lastly, we observed that sites labeled by either [^{3}H](+)pentazocine or [^{3}H]DTG do not respond to P450 induction (Table 5), suggesting that neither σ subtype is related to these cytochrome P450 isoforms.

The hypothesis that the σ recognition site may be cytochrome P450 was supported by data from Ross [11], who observed that the P450 inhibitor proadifen demonstrated nanomolar affinity for the σ site labeled by [^{3}H](+)SKF-10,047 in rat brain and liver. Subsequent studies by Ross [28] and Klein *et al.* [31] confirmed the inhibitory activity of proadifen at σ sites labeled by [^{3}H]haloperidol [28], [^{3}H](+)3-PPP [28, 31], and [^{3}H]dextromethorphan [31]. Proadifen labeled high- and low-affinity sites for [^{3}H]DTG binding in guinea pig brain, with K_i values of 4 and 780 nM [31]. We observed that proadifen was a potent inhibitor of [^{3}H](+)pentazocine binding (Table 2), but was less potent in inhibiting [^{3}H]DTG binding in all tissues examined (Table 3). In rat, the affinity of proadifen was 0.2 to 0.7 μM in liver and 0.8 to >10 μM in brain [28, 56], suggesting species differences in the response to proadifen. GBR-12909 potently inhibited the binding of [^{3}H]DTG in all three tissues, and was 5-fold less potent in liver compared with brain and testes (Table 3), while GBR-12909 was 5-fold less potent in inhibiting [^{3}H](+)pentazocine binding in liver and testes compared with brain (Table 2). Lobeline was equipotent in brain and testis for both σ ligands, 2-fold less potent in inhibiting [^{3}H](+)pentazocine binding in liver, and 2-fold more potent in inhibiting [^{3}H]DTG binding in liver. In general, certain P450 substrates and inhibitors, such as proadifen, lobeline and GBR-12909, inhibited σ binding, whereas others, such as quinidine and sparteine, did not.

Our data failed to establish a correlation between the σ binding site and cytochrome P450IID1 based on a comparison of the affinities of various compounds for the σ binding site in guinea pig liver versus the affinities of the same compounds to inhibit the enzyme in human liver (Table 4) [47]. Quinidine, a specific inhibitor of cytochrome P450IID1, exhibited a K_i value of 66 nM for the enzyme [47], but was a weak inhibitor of σ binding in all tissues except testis. The lack of correlation of σ binding with inhibitory potency at the enzyme suggests that there may be similarities in the pharmacophore for both cytochrome P450 and the σ recognition site, and our data support the notion that the affinities of certain P450 compounds for the σ site are due to structural similarities that render them potent at the σ site.

The lack of up-regulation of σ sites in liver following induction of cytochrome P450 by phenobarbital or β-naphthoflavone indicated that the σ site is not related to cytochrome P450IIB1 or P450IA1. In spite of a 3- to 4-fold increase in activity of these enzymes in liver, no significant increase was observed in the B_{max} for σ sites labeled by either ligand. Basile *et al.* [56] observed a significant increase in the B_{max} for [^{3}H]DTG binding in liver of rats treated with either phenobarbital or 3-methylcholanthrene, and this effect occurred in whole homogenate and in every subcellular fraction examined except for the nuclear fraction. The differences between our results and those of Basile *et al.* [56] may be due to differences

in the amount of enzyme induction achieved, or in the compound used to induce cytochrome P450IA1. Since no enzyme activity data were reported in the Basile study [56], it is unknown if greater enzyme induction was achieved with their protocol, which may have led to the significant increase in [^{3}H]DTG binding reported in their study.

Other studies on σ receptor regulation following pharmacological manipulation of cytochrome P450 levels have yielded conflicting results. Knight *et al.* [33] treated rats with spironolactone, a steroid that depletes cytochrome P450 in rat testis [57]. Although the number of sites labeled by [^{3}H]DTG was decreased significantly in liver and brain, no alteration of σ binding was observed in testis, where spironolactone exerts its greatest effects in rat [57] and where significant numbers of σ sites are present (Figs. 1 and 2 and [15]). Dwoskin and coworkers [34] examined the binding of the σ ligand [^{3}H](+)3-PPP in brain and liver of the Dark Agouti rat strain, which lacks the gene product for cytochrome P450IID1. The number of σ binding sites in Dark Agouti rats was decreased by 27% in brain and by 26% in liver compared with controls, suggesting that at least some σ sites in these tissues may be associated with cytochrome P450IID1. However, [^{3}H](+)3-PPP has direct effects on dopaminergic systems [see Ref. 58] as well as affinity for σ sites [10]. Since compounds with activity at the dopamine transporter also inhibit cytochrome P450IID1 in brain [29, 30], these studies in Dark Agouti rats should be repeated with a selective σ ligand such as [^{3}H](+)pentazocine [14].

The lack of effect that we and others [56] observed on σ binding and cytochrome P450 induction in brain is not surprising, given several reports demonstrating that the ability to induce P450 in brain is limited. Guengerich and Mason [36] reported a 1.5-fold induction with phenobarbital or 3-methylcholanthrene treatment, and a 19-fold increase in activity for 7-ethoxycoumarin-*O*-deethylase. Measurable amounts of 7-ethoxycoumarin-*O*-deethylase activity were reported for whole brain in amounts of 1.6 pmol/min/mg [39]. Induction with β-naphthoflavone was observed by the inhibitory effects of antibodies on 7-ethoxycoumarin-*O*-deethylase activity but was not altered in a microsomal preparation. Anandatheerthavarada *et al.* [41] reported a 2-fold increase in 7-ethoxycoumarin-*O*-deethylase activity with 3-methylcholanthrene induction and no effect with phenobarbital induction, but Warner *et al.* [40] reported that the P450 content in brain is too low to permit reproducible quantification in microsomal preparations. In our hands, the amount of P450 activity in brain was below the limit of detection of our 7-ethoxycoumarin-*O*-deethylase assay and may be due to the limited inducibility of P450 in brain, and the absence of up-regulation of σ binding sites following enzyme induction in either brain or liver suggests that the σ binding site and cytochrome P450 are unrelated. Alternatively, other variables that may have influenced these results are possible differences between σ ligands in affinities for various P450 isoforms, or the heterogeneity of various P450 isoforms in brain regions. Although it is known that total P450 exhibits a selective distribution in brain [40], the relative amounts of P450 isoforms in brain and their regional distributions are unknown. There is also cross-reactivity among isozymes with respect to their inducibility, such that P450IIB2 and P450IIC6 are induced by phenobarbital, and P450IIE1 and P450IA2 are induced by β-naphthoflavone [44]. This lack of selectivity for inducers of cytochrome P450 may obscure any selectivity of σ ligands for a particular P450 isoform. A direct comparison of staining for antibodies selective for particular isozymes versus σ binding autoradiography in discrete regions of brain would aid in resolving the issue.

In conclusion, these results suggest that σ sites in the periphery are similar to those in brain, and that the σ binding site is not identical with cytochrome P450IIA1, P450IA1 or P450IID1. It is uncertain at present whether some σ ligands serve as substrates of cytochrome P450 or as inhibitors of the enzyme, and the extent to which certain σ ligands may be selective for isoforms of P450 is unknown. The recent observation that chronic administration of nicotine can induce P450 in brain [59] but not liver suggests that the function of P450 in brain and its susceptibility to induction by different agents may differ from that in the periphery. A systematic investigation of the effects of selective σ ligands on specific cytochrome P450 isozymes may establish a firm relationship between the two that would elucidate the true identity of the σ recognition site.

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