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Pharmacological Analysis of Sterol Δ8-Δ7 Isomerase Proteins with [^{3}H]Ifenprodil

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ABSTRACT

Sterol Δ8-Δ7 isomerases (SIs) catalyze the shift of the double bond from C_{8-9} to C_{7-8} in the B-ring of sterols. Surprisingly, the isoenzymes in fungi (ERG2p) and vertebrates [emopamil binding protein (EBP)] are structurally completely unrelated, whereas the σ_1 receptor, a mammalian protein of unknown function, bears significant similarity with the yeast ERG2p. Here, we compare the drug binding properties of SIs and related proteins with [^{3}H]ifenprodil as a common high affinity radioligand (K_d = 1.4–19 nM), demonstrating an intimate pharmacological relationship among ERG2p, σ_1 receptor, and EBP. This renders SIs a remarkable example for structurally diverse enzymes with similar pharmacological profiles and the propensity to bind drugs from different chemical groups with high affinity. We identified a variety of experimental drugs with nanomolar affinity for the human EBP (K_i = 0.5–14 nM) such as MDL28815, AY9944, triparanol, and U18666A. These compounds, as well as the fungicide tridemorph and the clinically used drugs tamoxifen, clomiphene, amiodarone, and opipramol, inhibit the *in vitro* activity of the recombinant human EBP (IC_{50} = 0.015–54 μM). The high affinity of the human EBP for ^{3}H-tamoxifen (K_d = 3 ± 2 nM) implies that the EBP carries the previously described microsomal antiestrogen binding site. Interactions of the EBP with structurally diverse lipophilic amines suggest that novel compounds of related structure should be counterscreened for inhibition of the enzyme to avoid interference with sterol Δ8-Δ7 isomerization.

SIs shift the Δ8-bond in the B-ring of sterols to C_{7-8}. In plants and mammals, the Δ7-bond then is removed by a Δ7-sterol reductase. The outstanding biological and medical significance of these last steps of cholesterol biosynthesis for morphogenesis is illustrated by the inborn Δ7-sterol reductase deficiency that causes a variable combination of malformations (Smith-Lemli-Opitz syndrome; Fitzky *et al*., 1998). There are currently two types of SIs, with molecular masses of 25–27 kDa (Moebius *et al*., 1997b). Strikingly, neither their amino acid sequences nor their transmembrane topologies are related. The yeast ERG2p is anchored in the membrane of the endoplasmic reticulum by an amino-terminal transmembrane segment, whereas the mammalian EBP has four putative transmembrane α-helices (Moebius *et al*., 1997b). The ERG2p is present in fungi such as *Saccharomyces cerevisiae* (GenBank accession number M74037), *Ustilago maydis* (Z17311), *Magnaporthe grisea* (Z22775), and *Neurospora crassa* (U59671). EBP was cloned from *Homo sapiens* (Z37986), *Mus musculus* (X97755), and *Cavia porcellus* (Z37985). The existence of SI in mammals (EBP) that is unrelated to the yeast isoenzyme (ERG2p) suggests that both enzymes evolved independently (Moebius *et al*., 1997b). Intriguingly, a mammalian protein that is structurally related to the ERG2p carries the high affinity (+)-[^{3}H]pentazocine binding site described previously as σ_1 receptor but exhibits no SI activity upon heterologous expression in yeast (Hanner *et al*., 1996). We already demonstrated an intimate pharmacological relationship between the ERG2p and the σ_1 receptor (Moebius *et al*., 1996, 1997a), which we now extend to the structurally unrelated EBP.

Ifenprodil used as a radioligand exerts protective effects in animal models of cerebral ischemia and is known to interact with *N*-methyl-D-aspartate receptors, σ receptors, and α_1-adrenoceptors (Benavides *et al*., 1992; Hashimoto and London, 1993; Priestley *et al*., 1995; Gallagher *et al*., 1996; Kasiwagi *et al*., 1996). Here, we address the following questions by using heterologous protein expression in *S. cerevisiae*: (1) Are the structurally diverse SI proteins from fungi (ERG2p) and mammals (EBP) pharmacologically related? (2) Are high affinity ligands of the human EBP also inhibitors of its cat-

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ABBREVIATIONS: SI, sterol Δ8-Δ7 isomerase; EBP, emopamil binding protein; ERG2p, sterol Δ8-Δ7 isomerase of *S. cerevisiae*; k_{+1} and k_{-1}, association and dissociation rate constant, respectively.

alytic activity? Our work establishes a detailed pharmacological profile of the human SI, an enzyme of considerable medical significance.

Experimental Procedures

Materials. (+)[^{3}H]Pentazocine (32 Ci/mmol), [^{3}H]ifenprodil (44 Ci/mmol), and [^{3}H]tamoxifen (85 Ci/mmol) were obtained from NEN (Vienna, Austria). Zuclomiphene, enclomiphene, triparanol, MDL5332, and MDL28815 [M-[(1,5,9)-trimethyldecyl]-4,10-dimethyl-8-aza-*trans*-decal-3β-ol] were from Hoechst Marion Roussel Research Institute (Cincinnati, OH). L690404 [1-butyl,4-dihydrospiro[naphthalene-1-(2*H*),4′-piperidine]; compound 25; Chambers *et al*., 1992] was from Merck Sharp & Dohme (Harlow, England). Fenpropimorph and tridemorph were from BASF (Limburgerhof, Germany). AY9944 [1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane] was from Dr. P. Benveniste (Strasbourg, France). Trifluperidol was from RBI (Natick, MA). U18666A [3β-(2-diethylaminoethoxy)-androstenone] was from BIOMOL (Hamburg, Germany). Opipramol was from ICI (Vienna, Austria) BM15766 [4-(2-[4-(4-cinnamyl)piperazine-1-yl]-ethyl)-benzoic acid] was from Boehringer Mannheim (Mannheim, Germany). Bradford Protein Reagent was from BioRad (Vienna, Austria). All other chemicals were obtained from Sigma (Vienna, Austria). *S. cerevisiae* strain WA0 was kindly provided by Dr. M. Bard (Indianapolis, IN). Strain JB811 was from Dr. K. Nasmyth (Vienna, Austria).

Binding assays. We incubated 0.6 nM (+)-[^{3}H]pentazocine or [^{3}H]ifenprodil in 0.25 or 0.5 ml of 25 mM Tris·HCl (pH 9 at 4°, pH 8.3 at 22°) for 16 hr at 22° with 2–35 μg/ml microsomal protein. Nonspecific binding was measured in the presence of 1 μM concentration of unlabeled drug. Serial dilutions of competing drugs were prepared in dimethylsulfoxide (Moebius *et al*., 1993) and added directly to the assay. The final dimethylsulfoxide concentration was ≤1%, which did not affect specific binding. For the separation of bound and free ligands, samples were filtered through Whatman GF/C filters presoaked in 0.3% (w/v) polyethyleneimine. Filters were washed with 10 mM ice-cold Tris·HCl (pH 9 at 4°). [^{3}H]Tamoxifen (0.6 nM) was incubated in 1 ml of 25 mM Tris·HCl (pH 8 at 4°, pH 7.3 at 22°) for 12 hr. Bound and free ligands were separated as described previously (Moebius *et al*., 1993). Binding parameters were obtained by nonlinear curve fitting to a rectangular hyperbola (K_d, B_{max}) or the general dose-response equation (IC_{50}, slope factors; DeLean *et al*., 1978). K_i values were calculated according to Linden (1982).

Cloning of the murine EBP. A mouse-liver cDNA library was prepared and screened as described previously (Hanner *et al*., 1995). The DNA sequence of the isolated clone was identical with GenBank clone X97755 (Silve *et al*., 1996). The open reading frame expression vector was constructed, introducing *Hin*dIII and *Not*I restriction sites and removing the 5′ and 3′ noncoding regions by polymerase chain reaction as described previously (Hanner *et al*., 1995).

Membrane preparation. Microsomes from yeast strain WA0 (*a his7–2 leu2–3, 112 ura3–52 erg2–3*) overexpressing the σ_1 receptor (6 × HIS-lamdaGP8-ORF; Hanner *et al*., 1996) or the human, mouse, and guinea pig EBP (Hanner *et al*., 1995) and from strain JB811 (*ade2–1 leu2–3, 112 pep4–3 trp1–289 ura3–52*) were prepared as described previously (Moebius *et al*., 1996). Guinea pig liver and whole brain microsomes were prepared by homogenization with a glass-Teflon homogenizer in 0.25 M ice-cold sucrose/10 mM Tris-HEPES, pH 7.4 (4°). The homogenate was centrifuged at 8,000 × *g*, and the resulting supernatant was collected by centrifugation at 100,000 × *g*. After a wash with 0.5 M KCl, 0.15 M Tris·HCl, pH 8.0 (4°), and centrifugation at 100,000 × *g*, the final pellet was resuspended in 5% (w/v) glycerol/20 mM Tris·HCl, pH 9 (4°), at a protein concentration of 4–8 mg/ml, shock-frozen in liquid nitrogen, and stored at −80°. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as a standard.

Determination of SI activity. For enzyme-inhibition experiments, 0.25–0.50 mg/ml microsomal protein from WA0 cells expressing the human EBP were incubated anaerobically in 100 mM potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 140 mM glucose, 10 mM glutathione, and 0.5 mM EDTA for 1.5 hr at 37° with 50 μM zymosterol in the presence or absence of drugs in a final volume of 1 ml as described previously (Paik *et al*., 1986). Zymosterol (5α-cholesta-8,24-dien-3β-ol) was prepared as described previously (Paik *et al*., 1986). Lipids were saponified by the addition of 1 ml of 25% (w/v) KOH in 95% (v/v) ethanol, and sterols were extracted with 8 ml of petroleum ether. Samples were evaporated to dryness, resuspended in 0.1 ml of chloroform, and subjected to gas-liquid chromatography. Sterols were quantified relative to an internal 5α-cholestane standard. Enzyme assays with liver microsomes from rats fed an enzyme-inducing diet were performed as described previously (Kang *et al*., 1995).

Results

[^{3}H]Ifenprodil binds to recombinant SIs. Because [^{3}H]ifenprodil binding studies in crude microsomes are hampered by the presence of multiple binding sites (see below), we used the yeast expression system described previously (Hanner *et al*., 1995, 1996; Moebius *et al*., 1997a). To get rid of the endogenous [^{3}H]ifenprodil binding activity of yeast (Moebius *et al*., 1996), SI proteins were expressed in *S. cerevisiae* strain WA0 (erg2–3) devoid of endogenous ERG2p (Moebius *et al*., 1996). [^{3}H]Ifenprodil binding to microsomes isolated from ERG2p, EBP, and σ_1 receptor expressing strains was variable (Fig. 1A) due to different expression levels (B_{max} = 15–71 pmol/mg of microsomal protein) and dissociation constants (K_d =1.4–19 nM) (Table 1). Kinetic studies revealed 8–25-fold differences in the association (k_{+1} = 4–100 10^3 M^{-1} sec^{-1}) and dissociation (k_{-1} = 3–25 10^{-5} sec^{-1}) rate constants (Table 1). The pH dependency of [^{3}H]ifenprodil binding to the human EBP was bell shaped, whereas the ERG2p and the σ_1 receptor shared sigmoid curves (Fig. 1, B and C). The [^{3}H]ifenprodil binding domains of all sterol Δ8-Δ7 isomerase proteins were sensitive to the divalent cations Zn^{2+} and Cu^{2+} (Table 1). SI proteins have in common high affinity for (−)-emopamil (K_i = 10–74 nM), opipramol (K_i = 2–47 nM), amiodarone (K_i = 11–62 nM), and L690404 (K_i = 1–4.7 nM) and low affinity for (+)-verapamil (K_i = 890–15,100 nM). The values reported here confirm results obtained previously with (−)-[^{3}H]emopamil and (+)-[^{3}H]pentazocine (Hanner *et al*., 1995, 1996), suggesting that

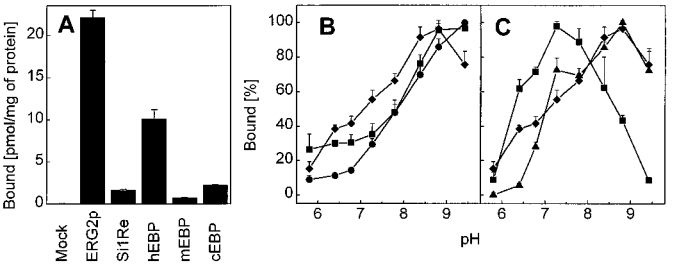

Fig. 1. A, [^{3}H]Ifenprodil binding to microsomes from yeast strain WA0 transformed with the shuttle plasmid YEp351ADC1 (Hanner *et al*., 1995, 1996; Moebius *et al*., 1996) without insert (*Mock*), carrying the DNAs encoding ERG2p of *S. cerevisiae* (*ERG2p*), the σ_1 receptor of *C. porcellus* (*Si$_1$Re*), and EBP of *H. sapiens* (*hEBP*), *M. musculus* (mEBP) and *C. porcellus* (cEBP). Microsomes were obtained as described (Moebius *et al*., 1996). B, pH dependency of [^{3}H]ifenprodil binding to the ERG2p of *S. cerevisiae* (●) the σ_1 receptor of *C. porcellus* (■), and the EBP of *C. porcellus* (♦). C, pH dependency of [^{3}H]ifenprodil binding to the EBP of *H. sapiens* (■), *M. musculus* (▲), and *C. porcellus* (♦). Data shown are the mean ± standard deviation of three experiments. pH values are given of the incubation mixture at 22°.

the binding domain for ifenprodil is identical to the binding domains for (−)-emopamil (EBP) and (+)-pentazocine (σ_1 receptor), respectively.

Haloperidol and ditolylguanidine discriminate two binding sites in guinea pig liver and brain microsomes. To investigate the association of native [^{3}H]ifenprodil acceptor sites with SI proteins, we characterized the pharmacological profile of [^{3}H]ifenprodil binding to guinea pig liver (Fig. 2A) and brain (Fig. 2B) microsomes, which contain high densities (B_{max} = 42 and 7.6 pmol/mg of microsomal protein; Table 2) of these sites (K_d = 1.9–2.5 nM, Table 2). The majority of brain [^{3}H]ifenprodil binding sites (84–88%, Table 2) showed high affinity for haloperidol (IC_{50} = 11 nM) and ditolylguanidine (IC_{50} = 27 nM), which is in agreement with the previously suggested binding of [^{3}H]ifenprodil to σ sites (Hashimoto and London, 1993). In liver microsomes, the proportion of low affinity haloperidol and ditolylguanidine binding sites was substantially higher (30–34%, Table 2) than in the brain. Their affinity for both drugs (IC_{50} = 141 and 4,500 nM, respectively, Table 2) was similar to the K_i determined for the recombinant guinea pig EBP (K_i = 250 and 10,600 nM, respectively, Table 3). This suggests that in liver, the haloperidol-insensitive [^{3}H]ifenprodil binding sites are associated with the EBP.

ERG2p and EBP share high affinity for SI inhibitors. Previous attempts to determine the affinity of drugs for postsqualene sterol biosynthetic enzymes were hampered by the sequential order of the *in vivo* enzymatic steps (Lewis *et al*., 1995) and by technically demanding *in vitro* assays. To determine the affinity of isomerization inhibitors for EBP, we measured the K_i values of the ^{3}H-ifenprodil-labeled human EBP for drugs that interfere with sterol biosynthesis *in vivo* or *in vitro* (Table 4). This substantially increased the number of structurally distinct compounds with high affinity for the ERG2p and the EBP, as well as for the σ_1 receptor (Moebius *et al*., 1996, 1997a). All SI proteins have in common high affinity for the morpholine fungicide tridemorph (K_i = 0.04–1.3 nM), the azadecalin MDL28815 (K_i = 0.44–0.58 nM), the experimental inhibitor of postsqualene cholesterol biosynthesis AY9944 (K_i = 0.5–12 nM), the cholesterol-lowering drug triparanol (K_i = 1.5–14 nM), the estrogen receptor agonist zuclomiphene (K_i = 1.6–4.7 nM), the aminosteroid U18666A (K_i = 0.1–3.3 nM), and the experimental antiestrogen MDL5332 (K_i = 0.67–54 nM). They also share low affinity for the inhibitor of the squalene-2,3-epoxidase naftifine (K_i = 310-1500 nM) and the Δ7-sterol reductase inhibitor BM15766 (K_i = 680–61,700 nM). The only major discrepancy was found for the antiestrogens tamoxifen and nafoxidine, which both have low affinity for the ERG2p (K_i = 1,470 and 232 nM, respectively) but high affinity for the EBP and the σ_1 receptor (K_i = 0.9–34 nM). Except for MDL28815, all drugs completely inhibited [^{3}H]ifenprodil binding to SI proteins with apparent slope factors close to unity (Table 4) as expected for competitive interaction. Competitive inhibition of [^{3}H]ifenprodil binding by tamoxifen was confirmed by an increased K_d value (control: K_d = 9.1 nM, B_{max} = 96 pmol/mg; 20 nM tamoxifen: K_d = 31 nM, B_{max} = 112 pmol/mg) of [^{3}H]ifenprodil for the human EBP in the presence of tamoxifen. The apparent slope factor for MDL28815 inhibition of [^{3}H]ifenprodil binding to the human EBP was 2.27 ± 0.26 (Table 4, three experiments). A possible explanation for a steep slope factor is that the receptor concentration exceeded the K_i value (Moebius *et al*., 1997a). For the MDL28815 inhibition experiments, this apparently was not the case (K_i = 0.5 ± 0.1 nM; R_T = 0.19 ± 0.01, three experiments). However, given the uncertainties of protein determination, we could not rule out higher receptor concentrations. To further clarify the mode of interaction between MDL28815 and the [^{3}H]ifenprodil binding site of the human EBP, we determined whether MDL28815 accelerated the ifenprodil (0.5 μM)-induced dissociation of the [^{3}H]ifenprodil-EBP complex. The dissociation rate constants in the absence or presence of 0.05 μM MDL28815 (60 ± 8 and 52 ± 12 10^{-6} sec^{-1}, respectively, three experiments), were essentially identical. We therefore conclude that MDL28815 is a competitive inhibitor with a K_i value of <0.5 nM.

[^{3}H]Tamoxifen binds to the EBP. The high affinity of the EBP for antiestrogens prompted us to investigate whether [^{3}H]tamoxifen binds to the recombinant human, murine, and guinea pig EBP (Fig. 3A). Indeed, the human protein showed high affinity for [^{3}H]tamoxifen (K_d = 3 ± 2 nM, four experiments; B_{max} = 240 ± 60 pmol/mg of microsomal protein, four experiments; Fig. 3B), whereas [^{3}H]tamoxifen binding activity was absent from mock transformed WA0 cells (Fig. 3A). Discrepancies of the B_{max} values for different radioligands ([^{3}H]ifenprodil: B_{max} = 71 pmol/mg; [^{3}H]tamoxifen: B_{max} = 240 pmol/mg) are observed frequently and most likely represent artifacts of the filtration assay [see Brauns *et al*. (1997) for discussion]. The affinity of the EBP for oxysterols such as 6-ketocholestanol (K_i = 0.11 ± 0.04 μM, three experiments), 7-hydroxycholesterol (K_i = 0.36 ± 0.06 μM, three experiments), and 7-ketocholesterol (K_i = 0.60 ± 0.23 μM, three experiments) was similar to the values reported previously for the antiestrogen binding site of liver microsomes (Hwang, 1990).

TABLE 1

Binding properties of [^{3}H] ifenprodil-labeled SI proteins

cDNAs encoding the guinea pig σ_1 receptor and EBP from humans, mouse, and guinea pig were expressed in yeast strain WA0 as described in Experimental Procedures.

	ERG2p *S. cerevisiae*	σ_1 Receptor *C. porcellus*	EBP		
			H. sapiens	*M. musculus*	*C. porcellus*
K_d (nM)	1.4[a]	9 ± 2	6 ± 2	19 ± 3	4 ± 2
B_{max} (pmol/mg)	61[a]	27 ± 3	71 ± 16	23 ± 6	15 ± 3
k_{+1} [10^3 M^{-1} × sec^{-1}]	45[a]	100 ± 7	43 ± 21	3.8 ± 1.4	14 ± 7
k_{-1} [10^{-6} sec^{-1}]	60[a]	250 ± 20	80 ± 10	30 ± 10	30 ± 10
Kinetic K_d (nM)	1.3	2.5	1.9	9	2
IC_{50} $ZnCl_2$ (μM)	4.4[a]	205 ± 25	0.6 ± 0.1	1.0 ± 0.2	1.1 ± 0.3
IC_{50} $CuCl_2$ (μM)	570[a]	10 ± 4	2.3 ± 0.5	1.1 ± 0.2	10 ± 4

Data shown are the mean ± standard deviation (n = 3).

[a] Data from Moebius *et al*., (1996).

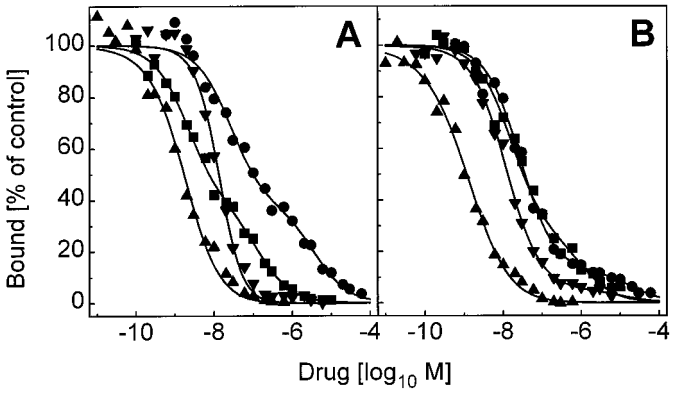

Fig. 2. Inhibition of [^{3}H]ifenprodil binding to guinea pig liver (A) and brain (B) microsomes by haloperidol (■), ditolylguanidine (●), ifenprodil (▲), and trifluoperazine (▼).

TABLE 2

Properties of the [^{3}H] ifenprodil binding sites in guinea pig brain and liver

Microsomes from guinea pig liver and brain were prepared as described in Experimental Procedures. Saturation analysis revealed B_{max} values of the liver and brain [^{3}H] ifenprodil binding sites of 42 ± 3 and 7.6 ± 0.2 pmol/mg of microsomal protein (n = 3) (K_d = 2.5 ± 0.8 and 1.9 ± 0.6 nM, respectively; (n = 3).

	Liver		Brain	
	IC_{50}	Slope	IC_{50}	Slope
	nM		*nM*	
Ifenprodil	2.4 ± 0.7	0.95 ± 0.15	1.7 ± 0.4	0.85 ± 0.05
Haloperidol	6.8 ± 2.8	0.64 ± 0.08[a]	23 ± 6	0.64 ± 0.04[a]
Ditolylguanidine	122 ± 33	0.55 ± 0.05[b]	36 ± 11	0.70 ± 0.09[b]
Trifluoperazine	11 ± 2	1.10 ± 0.13	13 ± 1	0.93 ± 0.06
(−) -Emopamil	28 ± 15	1.00 ± 0.12	34 ± 17	0.81 ± 0.09

Data shown are mean ± standard deviation (n = 3).
Biphasic fitting of the data (see Fig. 2) gave the following IC_{50} values.
[a] Haloperidol: Liver IC_{50} (high) = 2.9 ± 1.6 nM, IC_{50} (low) = 141 ± 67 nM (30 ± 6% of sites); brain IC_{50} (high) = 11 ± 8 nM, IC_{50} (low) = 1,720 ± 1,060 nM (16 ± 2% of sites).
[b] Ditolylguanidine: Liver IC_{50} (high) = 22 ± 8 nM, IC_{50} (low) = 4,500 ± 1,400 nM (34 ± 6% of sites); brain IC_{50} (high) = 27 ± 4 nM, IC_{50} (low) = 24,000 ± 8,000 nM (12 ± 2% of sites).

Inhibition of the *in vitro* activity of the human EBP by experimental and therapeutic drugs. Triparanol, zuclomiphene, and tamoxifen inhibit the mammalian SI *in vivo*, but only for AY9944 are *in vitro* affinities are known (Ramsey *et al.*, 1977; Paik *et al.*, 1986; Popják *et al.*, 1989; Gylling *et al.*, 1995). We therefore measured the IC_{50} value of drugs identified in the [^{3}H]ifenprodil binding assay for inhibition of the SI activity of the human EBP. All drugs that have high affinity for the [^{3}H]ifenprodil binding site (Table 4) also inhibit the SI activity of the recombinant human EBP. Similar results were obtained for the native SI from rat liver microsomes (data not shown). Except for MDL28815, slope factors were close to unity. As in the [^{3}H]ifenprodil binding assay, the steep slope factor of MDL28815 could reflect that the enzyme concentration (E_T) in the SI assay (E_T (estimated from the density of [^{3}H]ifenprodil binding sites) = 0.05 μM) exceeded the drug concentration (IC_{50}= 0.014 μM, Table 5). The 1000-fold discrepancy between the IC_{50} values for inhibition of enzymatic activity and the K_i values measured by [^{3}H]ifenprodil binding was intriguing (Table 5). We therefore examined the possible time dependence of inhibition assuming that the substrate-enzyme complex formed much more rapidly than the inhibitor-enzyme complex. Preincubation with 3 μM ifenprodil for 1, 2, or 3 hr had no effect on the extent of inhibition compared with a nonpreincubated sample (not shown). Next, we investigated whether the detergent required for suspension of the substrate inhibited the binding activity. Tyloxapol [0.15% (w/v)] almost completely abolished [^{3}H]ifenprodil binding (not shown). However, in the SI assay, much higher protein concentrations (0.25–0.5 mg/ml) than those in the binding assay (0.005–0.01 mg/ml) are used. The addition of microsomal carrier protein from a mock transformed yeast strain devoid of binding activity (Fig. 1A) partially restored radioligand binding (not shown). Saturation analysis with [^{3}H]ifenprodil revealed a 4.5-fold increase of the K_d value in the presence of 0.15% (w/v) tyloxapol and 0.4 mg/ml microsomal carrier protein (7.9 and 35 nM, respectively; Fig. 1A). Unexpectedly, zymosterol, the SI substrate, potently inhibited [^{3}H]ifenprodil binding (K_i = 500 ± 110 nM; slope factor = 1.11 ± 0.03; three experiments). Inhibition was due to an increase in the apparent K_d value, whereas the B_{max} value remained unchanged (Fig. 4B). The K_i values determined from either the Schild plot of the results from saturation experiments (K_i = 420 ± 130 nM; slope factor = 0.98 ± 0.17; three experiments) or the inhibition of ^{3}H-ifen-

TABLE 3

Pharmacological profiles of [^{3}H] ifenprodil-labeled SI proteins

cDNAs encoding the guinea pig σ_1 receptor and EBP from humans, mouse, and guinea pig were expressed in yeast strain WA0 as described in Experimental Procedures. Apparent slope factors were close to 1 (0.80–1.20) and were omitted for better clarity.

	K_i				
	ERG2p *S. cerevisiae*	σ_1 Receptor *C. porcellus*	EBP		
			H. sapiens	*M. musculus*	*C. porcellus*
	nM				
(+) -Emopamil	65[b]	13 ± 5	19 ± 4	77 ± 16	2.3 ± 0.6
(−) -Emopamil	74[a]	46 ± 4	20 ± 2	23 ± 6	10 ± 3
(+) -Verapamil	2,900 ± 500	15,100 ± 4,300	4,070 ± 1,120	3,630 ± 250	890 ± 110
(−) -Verapamil	>50,000	3,500 ± 1,100	2,690 ± 400	1,520 ± 100	2,900 ± 350
Trifluoperazine	500[a]	203 ± 4	3.6 ± 0.3	7.7 ± 2.0	3.9 ± 0.8
Opipramol	17[a]	1.9 ± 0.6	3.8 ± 1.2	47 ± 11	8.5 ± 0.5
Amiodarone	62[a]	11 ± 1	35 ± 6	60 ± 11	16 ± 1
Haloperidol	0.5[a]	1.6 ± 0.3	350 ± 30	435 ± 40	250 ± 40
Ditolylguanidine	1,980[a]	44 ± 13	14,400 ± 950	4,300 ± 850	10,600 ± 2,900
L690404	4.7[b]	2.5 ± 0.3	2.3 ± 0.3	N.D.	1.0 ± 0.1

Data shown are mean ± standard deviation (n = 3). N.D., not determined.
[a] Data from Moebius *et al.* (1996).
[b] data from Moebius *et al.* (1997a).

prodil binding at a single ligand concentration (see above) were essentially identical. From the K_i value of zymosterol, we estimated that at the concentration of zymosterol used in the enzyme inhibition experiments of 50 μM, the K_d value of ifenprodil was increased 100-fold. The K_m value (25 μM) for zymosterol was 100-fold higher than the K_i value of zymosterol in the ^{3}H-ifenprodil binding assay (0.25 μM). From the V_{max} value determined by kinetic analysis (0.325 nmol/min/mg protein) and the B_{max} value determined by radioligand binding (100 pmol/mg protein), we estimated the turnover rate of the SI ($k_3 = 5 \times 10^{-2}$ sec^{-1}). To clarify whether EBP ligands are competitive inhibitors of the sterol Δ8-Δ7 isomerase, we measured the kinetics of isomerization in the absence and presence of inhibitors. Except for MDL28815, which also had a minor effect on the K_m value, all drugs tested (ifenprodil, tamoxifen, and enclomiphene) changed the V_{max} value but not the K_m value (Fig. 4C).

TABLE 4

SI proteins share high affinity for inhibitors of postsqualene sterol biosynthesis and related drugs

cDNAs encoding the guinea pig σ_1 receptor and the human EBP were expressed in yeast strain WA0 as described in Experimental Procedures. The affinities of the yeast ERG2p were determined as described (Moebius *et al.*, 1996). Apparent slope factors close to 1 (0.80–1.20) were omitted for better clarity. Identical results as for the human EBP were obtained for the EBP from *C. porcellus* (not shown).

	K_i		
	ERG2p *S. cerevisiae* [^{3}H] Ifenprodil	σ_1 Receptor *C. porcellus* (+)-[^{3}H] Pentazocine	EBP *H. sapiens* [^{3}H] Ifenprodil
		nM	
Sterol isomerase inhibitors			
Fenpropimorph	0.05[a]	0.011[b]	44 ± 15
Tridemorph	0.09[a]	0.039[b]	1.3 ± 0.4
MDL28815	0.44[a]	0.48[b]	<0.54 ± 0.10[c]
AY9944	5.8[a]	0.50[b]	12 ± 5
Triparanol	1.5[a]	8.2[b]	14 ± 4
Tamoxifen	1,470[b]	34[b]	2.8 ± 1.0
Zuclomiphene	1.6[b]	4.7[b]	3.0 ± 0.3
Enclomiphene	164[b]	7.7[b]	0.69 ± 0.11
Trifluperidol	0.15[b]	0.83[b]	354 ± 50
Sterol biosynthesis inhibitors			
U18666A	0.10 ± 0.02	1.1 ± 0.3	1.9 ± 0.8
BM15766	16,480 ± 3,140	680 ± 90	34,000 ± 8,200
Naftifine	310 ± 25	880 ± 40	1,500 ± 40
Terbinafine	>50,000	N.D.	>50,000
Tamoxifen analogues			
MDL5332	0.67 ± 0.24	3.2 ± 1.3	54 ± 27
Nafoxidine	232 ± 101	30 ± 14	0.9 ± 0.3

Data shown are mean ± standard deviation (n = 3). N.D., not determined.
[a] data taken from (Moebius et al., 1996)
[b] data taken from (Moebius et al., 1997a)
[c] apparent slope factor 2.27 ± 0.26.

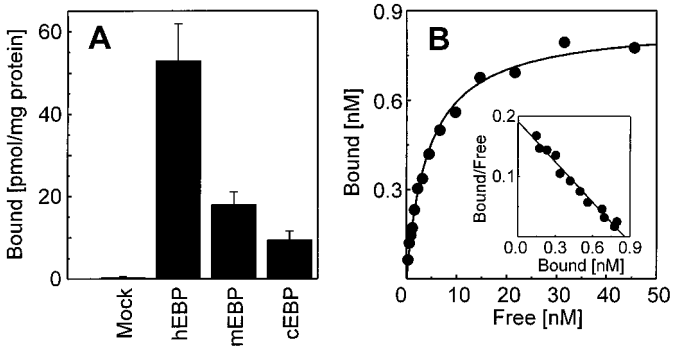

Fig. 3. A, [^{3}H]Tamoxifen binding to microsomes from yeast strain WA0 transformed with the shuttle plasmid YEp351ADC1 (Hanner *et al.*, 1995; Moebius *et al.*, 1996) without insert (*Mock*), carrying the DNAs encoding EBP of *H. sapiens* (*hEBP*), *M. musculus* (*mEBP*), and *C. porcellus* (*cEBP*). Microsomes were obtained as described previously (Moebius *et al.*, 1996). B, Saturation of 6 μg/ml of yeast microsomes expressing the human EBP with tamoxifen giving a K_d value of 5.0 nM and a B_{max} value of 0.90 nM protein. *Inset*, linear transformation of the data.

Discussion

[^{3}H]Ifenprodil is a high affinity ligand for SI proteins. In our previous studies characterizing EBP and σ_1 receptor, we used the structurally distinct radioligands [^{3}H]emopamil (Hanner *et al.*, 1995) and (+)-[^{3}H]pentazocine (Hanner *et al.*, 1996), hampering the comparison of equilibrium and kinetic binding constants. We now establish [^{3}H]ifenprodil as a common high affinity ligand. Kinetic studies with [^{3}H]ifenprodil revealed different rate constants for the homologous EBPs from human and mouse (Table 1). In contrast to the nearly diffusion limited association rate constant of lovastatin for HMG-CoA-reductase ($k_{+1} = 3 \times 10^7$ M^{-1} sec^{-1}; Schloss, 1988, and references within), [^{3}H]ifenprodil binding to SI proteins is 1,000–10,000-fold slower ($k_{+1} = 4$–45×10^3 M^{-1} sec^{-1}). The molecular basis of such slow binding, which is frequently observed for compounds that mimic reaction intermediates (Schloss, 1988), is yet unknown and could reflect slow changes of protein conformation or of group protonization. Based on the divalent cation sensitivity and pH dependency of [^{3}H]ifenprodil binding, we propose histidine (pK_s = 6.5), aspartate (pK_s = 4.4), glutamate (pK_s = 4.4), or cysteine (pK_s = 8.5) residues to be in the vicinity of the drug binding site.

EBP carries the microsomal antiestrogen binding site. The previously described high affinity (K_d = 1–2 nM) microsomal binding site for the antiestrogen [^{3}H]tamoxifen (Watts and Sutherland, 1984; Clark *et al.*, 1987; Hwang, 1990) is suggested to be associated with the EBP for the several reasons. (1) The two sites have identical affinities for a variety of structurally diverse drugs (tamoxifen, triparanol, trifluoperazine, MDL5332, nafoxidine, and U18666A/MDL5341; Clark *et al.*, 1987). (2) The sites have similar tissue distributions (liver > adrenal gland > kidney > lung;

TABLE 5

Inhibition of [^{3}H] ifenprodil binding and catalytic activity of the human EBP.

K_i or K_d values were taken from Table 1, 2, and 4. SI activity was measured as described in Experimental Procedures in the absence or presence of drug.

	K_i or K_d	IC_{50}	Apparent slope factor
	nM	μM	
Amiodarone	35	54 ± 5	0.72 ± 0.11
AY-9944	12	16 ± 6	0.69 ± 0.12
(±) -Emopamil	19	7 ± 1	0.83 ± 0.22
Enclomiphene	0.7	0.3 ± 0.1	0.68 ± 0.07
Ifenprodil	6	14 ± 4	0.62 ± 0.18
MDL5332	54	45 ± 3	0.75 ± 0.12
MDL28815	0.5	0.014 ± 0.001	2.22 ± 0.32
Nafoxidine	0.9	0.5 ± 0.1	0.94 ± 0.11
Opipramol	3.8	6 ± 1	0.82 ± 0.10
Tamoxifen	2.8	1.8 ± 0.1	0.65 ± 0.07
Tridemorph	1.3	3 ± 0.4	0.88 ± 0.03
Trifluoperazine	3.6	8 ± 2	0.85 ± 0.07
Triparanol	14	7 ± 3	0.67 ± 0.07
U18666A	1.9	4 ± 1	1.11 ± 0.02
Zuclomiphene	3.0	2.0 ± 0.1	0.71 ± 0.16

Data shown are mean ± standard deviation (n = 3). Given in parenthesis are the SI activities in percent of control determined in the presence of 100 μM BM15766 (97 ± 5), fenpropimorph (40 ± 4), haloperidol (50 ± 8), trifluperidol (45 ± 10), 6-ketocholestanol (36 ± 10), and 7-OH-cholesterol (61 ± 5).

Hwang, 1990; Moebius *et al.*, 1993). (3) The sites have identical densities in liver (30 pmol/mg of microsomal protein (Watts and Sutherland, 1984; Moebius *et al.*, 1993) and subcellular localizations in the endoplasmic reticulum (Watts and Sutherland, 1984; Moebius *et al.*, 1993).

Inhibition of the sterol Δ8-Δ7 isomerase by EBP ligands is noncompetitive. To account for the 1000-fold discrepancy between the K_i values determined in the binding assay and the IC_{50} values measured for inhibition of sterol Δ8-Δ7 isomerization (Table 5), we investigated the effect of the detergent tyloxapol required for resuspension of the substrate (Paik *et al.*, 1986) and of the substrate zymosterol itself on [^{3}H]ifenprodil binding. At the concentration of 0.15% (w/v) used in the SI assay, tyloxapol increased the K_d value 4.5-fold. Zymosterol potently inhibited [^{3}H]ifenprodil binding (K_i = 0.4–0.5 μM), suggesting that the concentration of zymosterol used in the SI inhibition experiments (50 μM) increased the K_d 100-fold. Taken together, the observations of a 4.5-fold increase of the K_d by the detergent tyloxapol and a 100-fold increase of the K_d by the substrate zymosterol explain why the IC_{50} values for inhibition of catalytic activity were 1000-fold higher than the K_i values determined by [^{3}H]ifenprodil binding. Zymosterol competitively inhibited [^{3}H]ifenprodil binding (Fig. 4B) in line with the hypothesis that SI inhibitors mimic the carbocationic reaction intermediate (Rahier and Taton, 1996) and thus bind within the catalytic cleft. To further confirm this assumption, we also determined the mode by which EBP ligands inhibit isomerization by kinetic analysis. Intriguingly, ifenprodil, tamoxifen, MDL28815, and enclomiphene reduced the V_{max} but (except for MDL28815) not the K_m value. This implies noncompetitive enzyme inhibition and apparently contradicts the assumption that the inhibitors mimic the carbocationic reaction intermediate. However, the same discrepancy was observed with a rationally designed inhibitor of the Δ7-sterol reductase (Rahier and Taton, 1996). This monoazasteroid (6-aza-B-homocholest-7-en-3β-ol) was synthesized as an analogue of a predicted carbocationic reaction intermediate but inhibited the maize Δ7-sterol reductase in a noncompetitive manner (Rahier and Taton, 1996). A possible explanation for this paradox is that the complexities of the assays for sterol biosynthetic enzymes (particulate enzyme and emulsified substrate) do not allow an interpretation of the inhibition kinetics (Rahier and Taton, 1996) or that the formation of the product occurs more rapidly than the dissociation of the enzyme-substrate complex, rendering substrate binding irreversible.

Structural implications of a common pharmacological profile. The [^{3}H]ifenprodil binding domains of SI proteins have in common nanomolar affinity for emopamil, ifenprodil, opipramol, L690404, amiodarone, MDL28815, AY9944, triparanol, zuclomiphene, MDL5332, and U18666A. These similarities raise some questions. (1) Why are the pharmacological profiles so intimately related? (2) Are SI proteins the only enzymes of postsqualene sterol biosynthesis that are high affinity drug binding proteins? (3) Which is the molecular basis of the propensity to bind a variety of chemically diverse compounds?

First, the complete lack of similarities between ERG2p and EBP with respect to their primary structures as well as their hydropathy plots is obvious (Moebius *et al.*, 1997b). Moreover, both enzymes differ in their reaction mechanism. In Fungi (through ERG2p) and mammals (through EBP), isomerization occurs through *cis* and *trans* proton addition and elimination, respectively (Moebius *et al.,* 1997b and references therein). Despite these fundamental differences, SI proteins share essentially identical pharmacological profiles. Drug binding to a regulatory domain common to SI proteins is conceivable, but no endogenous regulators of SI activity are known. The competitive inhibition of [^{3}H]ifenprodil binding by zymosterol, the similar pharmacological profiles of ERG2 and EBP, and the structural similarities of SI inhibitors with the carbocationic reaction intermediate (Rahier and Taton, 1996, and references therein) suggest that the inhibitor binding site and the catalytic domain overlap. This implies that amino acid residues required for binding of the sterol substrate or for the shift of the Δ8-bond also provide interaction sites for high affinity inhibitor binding. The [^{3}H]ifenprodil binding assay will be an excellent tool to test our hypothesis

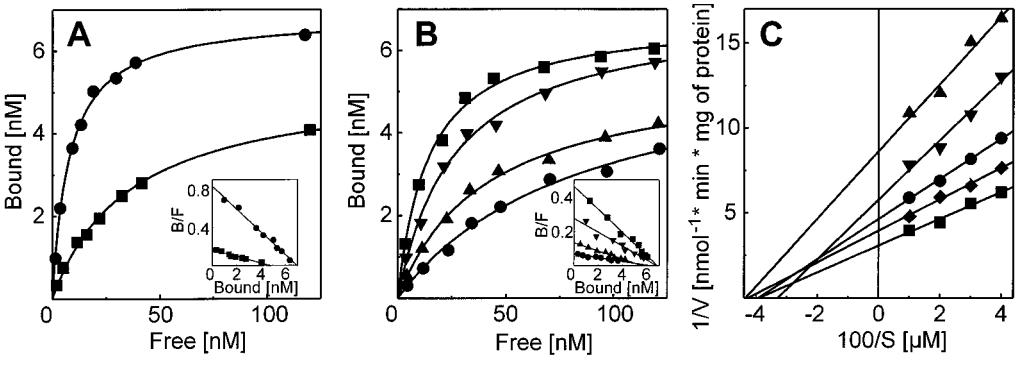

Fig. 4. Interactions among enzyme, substrate, and inhibitor. A, Saturation of the yeast-expressed human EBP with [^{3}H]ifenprodil in the absence (●) or presence (■) of 0.15% (w/v) tyloxapol. Incubation of 65 μg/ml microsomal protein from an EBP-expressing yeast strain was carried out in the absence (K_d = 7.9 nM, B_{max} = 102 pmol/mg protein) or presence of 0.15% (w/v) tyloxapol and 0.4 mg/ml microsomal carrier protein from a mock transformed yeast strain (K_d = 36 nM, B_{max} = 79 pmol/mg protein) at 22° for 12 hr. *Inset*, linear transformation of the data. B, Saturation of the yeast-expressed human EBP with ^{3}H-ifenprodil in the absence (■) or presence of 0.4 μM (▼), 1 μM (▲), and 2 μM (●) zymosterol. From the experiment shown, K_d values were determined (■, 14 nM; ▼, 27 nM; ♦, 41 nM; ●, 86 nM). *Inset*, linear transformation of the data (B/F, bound/free). From the Schild plot of the results from three separate experiments, a K_i value for zymosterol of 500 ± 110 nM and a slope factor of 0.98 ± 0.17 were obtained. C, Kinetics of EBP-mediated zymosterol Δ8-Δ7 isomerization in the absence (■, K_m = 25 μM; V_{max} = 0.325 nmol/mg/min) or presence of 3 μM ifenprodil (♦, K_m = 23 μM, V_{max} = 0.253 nmol/mg/min), 0.1 μM tamoxifen (●, K_m = 25 μM, V_{max} = 0.216 nmol/mg/min), 0.1 μM MDL28815 (▼, K_m = 30 μM, V_{max} = 0.173 nmol/mg/min), and 0.3 μM enclomiphene (▲, K_m = 23 μM, V_{max} = 0.116 nmol/mg/min). Essentially identical results were obtained with 5 μM ifenprodil (V_{max} = 0.188 nmol/mg/min), 0.2 μM MDL28815 (V_{max} = 0.159 nmol/mg/min), and 0.1 μM enclomiphene (V_{max} = 0.238 nmol/mg/min). The zymosterol concentration was varied with a constant final concentration of 0.3% (w/v) of tyloxapol. The length of incubation was limited to 1.5 hr.

of an intimate spatial and functional relationship between the catalytic cleft and the inhibitor binding domain by systematic site-directed mutagenesis in SI proteins.

Second, not only sterol Δ8-Δ7 isomerization but also the steps mediated by the Δ7-, Δ24-, and Δ14-sterol reductases involve putative carbocationic reaction intermediates (Rahier and Taton, 1996), in line with the overlapping pharmacological profiles of sterol reductases and isomerases. Ifenprodil and MDL28815 also inhibit the Δ14-sterol reductase (van Sickle *et al*., 1993; Moebius *et al*., 1996); trifluperidol, AY9944, and fenpropimorph inhibit the Δ7-sterol reductase (Kraml *et al*., 1964; Braun, 1969; Taton and Rahier, 1991; Moebius *et al*., 1998); and triparanol, trifluoperazine, and U18666A inhibit the Δ24-sterol reductase (Scallen *et al*., 1961; Filipovic and Buddecke, 1987; Bae and Paik, 1997). It is therefore intriguing that none of the sterol reductases was reported to also be a high affinity drug binding protein. Until recently, the primary structures of mammalian sterol-reductases were unknown. Cloning and heterologous expression of the human Δ7-sterol reductase (Moebius *et al*., 1998) paved the way to investigate whether this enzyme is also a high affinity drug binding protein and to clarify whether the different reaction mechanisms of sterol Δ8-Δ7 isomerization (delivery and receipt of a proton without cofactor requirement) and Δ7-sterol reduction (delivery of a proton by the enzyme and of a hydride ion by the cofactor NADPH) create different or similar environments for high affinity binding of lipophilic amines.

Third, the striking ability of SI proteins to bind a variety of structurally distinct drugs is unparalleled except for the multidrug resistance protein involved in the extrusion of xenobiotics. The multidrug resistance protein also takes part in cholesterol biosynthesis (Metherall and Huijan, 1996), suggesting that the propensity to bind structurally distinct compounds could be related to the presence of a sterol binding site in this protein.

Pharmacological and toxicological significance of SI inhibitors. Postsqualene cholesterol biosynthesis is pivotal for human ontogenesis. This is illustrated by malformations, failure to thrive, and mental retardation in children with the Smith-Lemli-Opitz syndrome due to a mutation in the Δ7-sterol reductase gene (Fitzky *et al*., 1998). The anticancer drug tamoxifen inhibited isomerization *in vitro* (IC_{50} = 1.8 μM) and compromised the SI activity in patients at daily doses of 40 mg (Gylling *et al*., 1995). Our data (Table 5) suggest that other drugs used at similar doses as tamoxifen might also inhibit the SI activity in humans. Among them are the antiarrhythmic amiodarone (IC_{50} = 54 μM; clinically used dose, 100–400 mg/day), the antidepressant opipramol (IC_{50} = 6 μM; dose, 100–300 mg/day), and the ovulation inducer clomiphene (IC_{50} = 0.3–2 μM; dose, 50–100 mg/day). The teratogenicity of SI inhibitors such as clomiphene and tridemorph in animals is established (Merkle *et al*., 1984; Schmidt *et al*., 1986), but the toxicological as well as the pharmacological significance of SI inhibition in humans remains to be clarified. The recent identification of intermediates of cholesterol biosynthesis other than 7-dehydrocholesterol (desmosterol and 8-dehydrocholesterol, respectively) in two children with the clinical characteristics of fatal Smith-Lemli-Opitz syndrome suggests that deficiencies of the Δ24-sterol reductase and the sterol Δ8-Δ7 isomerase, respectively, also result in a Smith-Lemli-Opitz syndrome-like phenotype (Clayton, 1998). Because of the ability of SI proteins to bind so many lipophilic amines, we recommend counterscreening of novel compounds with structural similarity to the drugs used in our study for interaction with the EBP and the σ_1 receptor. We previously suggested the EBP to be the target of anti-ischemic drugs because of its ability to bind compounds beneficial in animal models of stroke (Moebius *et al*., 1993). If inhibition of sterol Δ8-Δ7 isomerization prevented ischemic damage, potent SI inhibitors would be candidates for evaluation in animal models of cerebral hypoxia.

Ifenprodil and other sterol Δ8-Δ7 isomerization inhibitors identified in this work will become important probes with which to investigate the molecular mechanism and the pharmacological and toxicological significance of sterol Δ8-Δ7 isomerization in humans.

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