Novel 4-(4-Aryl)cyclohexyl-1-(2-pyridyl)piperazines as $\Delta_8-\Delta_7$ Sterol Isomerase (Emopamil Binding Protein) Selective Ligands with Antiproliferative Activity

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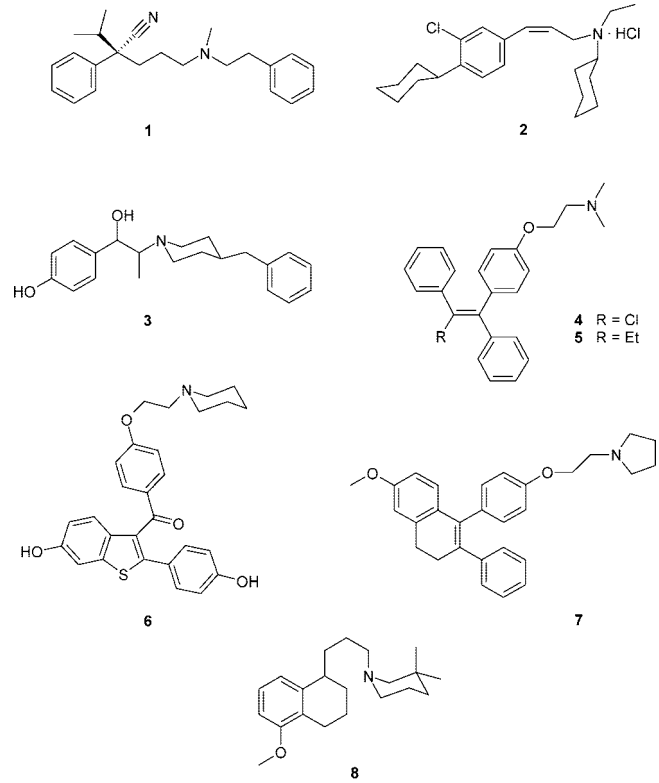
To find $\Delta_8-\Delta_7$ sterol isomerase (EBP) selective ligands, various arylpiperazines previously studied and structurally related to some σ receptors ligands were preliminarily screened. Consequently, a novel series of 2- or 2,6-disubstituted (CH_3, CH_3O, Cl, F) *cis*- and *trans*-4-(4-aryl)cyclohexyl-1-(2-pyridyl)piperazines was developed. Radioreceptor binding assays evidenced *cis*-**19**, *cis*-**30** and *cis*-**33** as new ligands with nanomolar affinity toward EBP site and a good selectivity relative to EBP-related σ receptors. The most selective 2,6-dimethoxy derivative (*cis*-**33**) demonstrated the highest potency ($EC_{50} = 12.9$ μM) and efficacy (70%) in inhibiting proliferation of human prostate cancer PC-3 cell line. Among the reference compounds, σ_2 agonist **36** (PB28) reached the maximum efficacy (100%), suggesting the contribution of the σ_2 receptor to the antiproliferative activity. This novel class of EBP inhibitors represents a valuable tool for investigating the last steps of cholesterol biosynthesis and related pathologies, as well as a starting point for developing new anticancer drugs.

Introduction

Postsqualene sterol biosynthetic pathway is an attractive target for blood cholesterol lowering. Inhibiting enzymes at the last steps of cholesterol biosynthesis can reduce the side-effects due to an early step inhibition such as at 3-hydroxy-3- methylglutaryl (HMG[a]) CoA-reductase level by vastatins. On the other hand, the inborn lack of enzymes acting in the final steps of cholesterol biosynthesis is known to generate rare but severe disorders. The discovery of specific inhibitors for such enzymes can help to better understand the biochemistry of these genetic defects.[1,2] CHILD (congenital hemidysplasia with ichthyosiform erythroderma and limb defects) syndrome,[3] Conradi-Hünermann-Happle syndrome (or X-linked dominant chondrodysplasia punctata type 2),[4,5] and Smith−Lemli−Opitz syndrome[6,7] are dysmorphogenetic syndromes of variable severity due to mutations to the genes encoding for (i) the C4-sterol dehydrogenase, (ii) the 3β-hydroxysteroid $\Delta^8-\Delta^7$ isomerase (EBP), and (iii) the 3β-hydroxysteroid Δ^7-reductase, respectively.[8] In the past, the clinical trials of Δ^7-reductase inhibitors such as 20,25-diazacholesterol and triparanol were discontinued because of their high toxicity.

EBP (i.e., Emopamil Binding Protein) is a vertebrate sterol $\Delta^8-\Delta^7$ isomerase which has demonstrated equal high binding affinity[9] for both enantiomers of emopamil, a Ca^{2+} channel blocker. EBP characterization was achieved using the anti-ischemic drug (−)-(*S*)-emopamil (**1**) (Chart 1).[10] Functionally, EBP catalyzes the shift of the double bond from C8−C9 to C7−C8 position in one of the last steps of cholesterol de novo biosynthesis. It has been hypothesized that in humans such isomerization occurs through a trans hydrogen addition−elimination reaction from 5α-cholesta-8,24-dien-3β-ol (zymosterol, **9a**) or dihydrozymosterol (**9b**) to give 5α-cholesta-7,24-dien-3β-ol (**10a**) or lathosterol (**10b**), respectively (Chart 2).[11] cDNA from human liver encoded EBP as a 230 amino acids, 27-kDa protein[9] spanning through four putative transmembrane domains.[12] EBP and its yeast counterpart ERG2p, which catalyzes the same step in yeast ergosterol biosynthesis, share a strikingly similar

Chart 1. Structures of Mammalian $\Delta_8-\Delta_7$ Sterol Isomerase (EBP) Ligands



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[a] Abbreviations: EBP, emopamil binding protein; PC-3, prostate cancer cells; HMG, 3-hydroxy-3-methylglutaryl; CHILD, congenital hemidysplasia with ichthyosiform erythroderma and limb defects; ERG2p, $\Delta_8-\Delta_7$ sterol isomerase of *Saccharomyces cerevisiae*; NMDA, *N*-methyl-D-aspartate; AEBS, antiestrogen binding site; 5-HT, 5-hydroxytryptamine; TMEDA, *N*,*N*,*N*,*N*-tetramethylethylendiamine; LDH, lactate dehydrogenase; ClogD, calculated logarithm of distribution; SI, sterol isomerase; RPMI, Roswell park memorial institute; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide.

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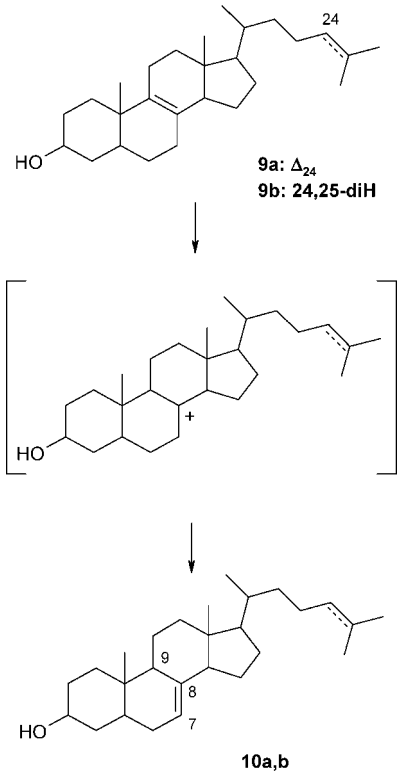
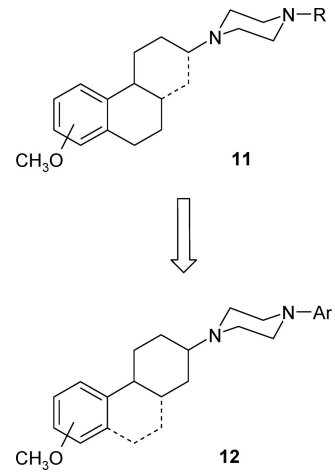
Chart 2. EBP-Catalyzed Isomerization



pharmacological profile and molecular mass, but their structures are unrelated. On the other hand, yeast ERG2p and human sigma-1 (σ_1) receptor share a 30% amino acid sequence, so that it has been proposed that σ receptors belong to the family of sterol isomerases.[12] Nevertheless, no enzymatic activity has been proven for σ_1 receptor, although several structural classes of ligands present both σ_1 and EBP pharmacological behavior. In addition, EBP ligands exert antiproliferative action in various tumoral cells, and the expression of the EBP protein together with the expression of the σ_1 receptor has been studied as a biomarker for prognostic purposes in breast carcinoma.[13]

The high affinity σ_1 receptor ligand *N*-[(2*Z*)-3-(3-chloro-4-cyclohexylphenyl)-2-propen-1-yl]-*N*-ethyl-cyclohexanamine hydrochloride (**2**, SR 31747A; Chart 1) was known to tightly bind also EBP and to exert antiproliferative effects.[14,15] Compound **2** elicited antitumoral activity partially through the inhibition of EBP isomerization activity.[16] Emopamil and related antiischemic drugs are obviously nonselective EBP ligands, as well as ifenprodil (**3**), another prototypic EBP ligand much studied, but presenting high affinities for NMDA site, σ_1 and α_1 receptors too.[17] Both emopamil and ifenprodil are noncompetitive inhibitors of EBP. Many other ligands have been proven to bind EBP, but most of them bind equally σ_1 receptor and others are drugs of various classes used in therapy. An exhaustive list is reported in a recent work, where different pharmacophores have been proposed for EBP and σ_1 receptor ligands, on the basis of molecular modeling techniques and virtual screening studies.[18] A high EBP affinity with moderate selectivity relative to σ_1 receptor was displayed by AEBS ligands, such as antitumor drugs enclomiphene (**4**) and tamoxifen (**5**).[18] A good selectivity was also displayed by estrogen receptor modulators raloxifene (**6**) and nafoxidine (**7**).[18] All these compounds inhibit isomerization activity of EBP. Nevertheless, high-affinity and selective EBP ligands are needed to better understand the role and importance of EBP function.

Chart 3. From Tetralin to Arylcyclohexyl Derivatives

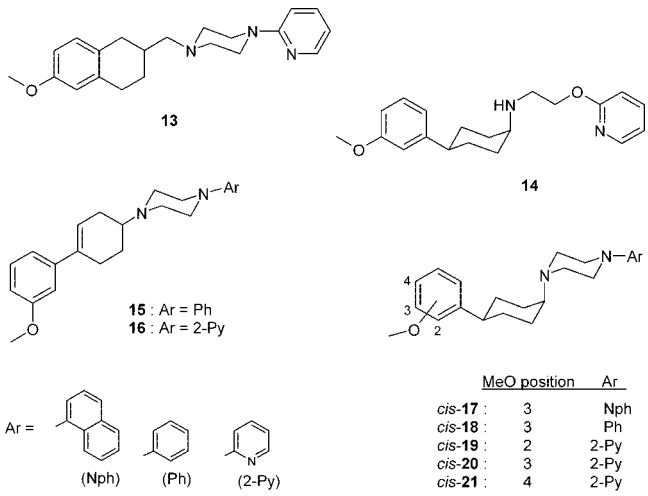


In an attempt of finding high-affinity and σ_1 and σ_2 selective ligands, we developed several Structure-Affinity Relationship (SAfiR) studies where the EBP affinity was also taken into account. Most of the σ_1 receptor ligands studied displayed fairly good to high affinity also toward EBP site, resulting in poor selectivities.[19−22] A good EBP selectivity was found for the high-affinity ligand 3,3-dimethyl-1-[3-(5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl)*n*-propyl]piperidine (**8**).[19] The tetralin or naphthalene moiety in our compounds appeared to be able to well mime both the benzomorphan template of σ_1 ligand (+)-pentazocine and the steroidal A and B rings of the EBP substrates **9a**,**b**. On the other hand, the A ring in these steroidal substrates is less planar than the tetralin nucleus in **11** (Chart 3), and the structures of specific EBP ligand **1** and related compounds do not present any bicycle nucleus. All things considered, we thought that such a bicycle might be unessential for EBP binding and the opening of the tetralin nucleus should lead to some specific EBP-site affinity. Furthermore, the intermediate propylenic chain, linked to the amine moiety, had to be constrained in a cyclohexyl ring in order to reduce the conformational freedom of the resulting structures **12**. We had followed a similar approach to prepare some arylpiperazine derivatives as serotonin 5-HT_{1A} receptor ligands.[23] Therefore, we screened a restricted series of these *N*-arylpiperazines and related compounds, choosing among the lowest-affinity ligands for serotonin 5-HT_{1A}, dopamine D_2, and adrenergic α_1 receptors, and testing them for EBP, σ_1 and σ_2 affinity (Chart 4). The results allowed to highlight the high-affinity and selective EBP ligand *cis*-4-[4-(2-methoxyphenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*cis*-**19**) as a lead compound (Table 1). Consequently, novel 2-pyridinylpiperazine derivatives were designed in order to investigate the importance of methoxyl substituent on the phenyl moiety and the role of electronic and steric effects of other substituents (Me, Cl, F) in determining the binding affinities. Some 2,6-disubstituted-phenyl derivatives (**32**−**35**) were prepared in order to hinder the free rotation of the phenyl ring linked to the cyclohexyl ring. The isomers of compound *cis*-**19** for the methoxyl position and for *cis*/*trans* geometry were tested to complete a SAfiR study. The best EBP ligands were then tested in antiproliferative assays on human prostate cancer PC-3 cell line.

Chemistry

The synthesis of final compounds **19**−**21** has already been reported.[23] The synthesis of all the novel target compounds **29**−**35** is depicted in the Scheme 1 and the intermediate keys

Chart 4. Structures of Preliminarily Screened Compounds

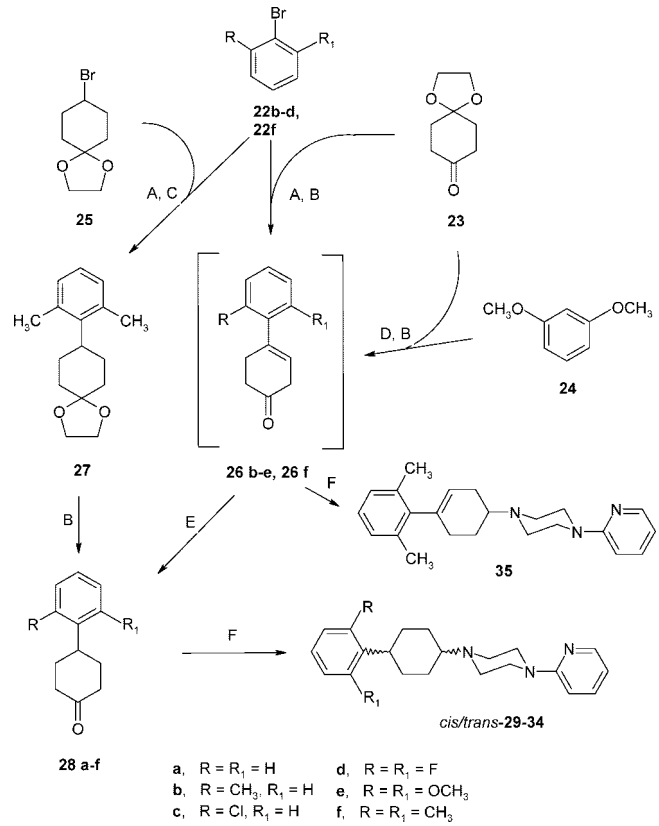


are ketones **26f** and **28a**−**f**. Starting from the appropriate bromobenzene derivatives **22b**−**d**, **f**, the corresponding Grignard's reagents were generated with Mg turnings and reacted with 1,4-cyclohexandione monoethylene ketal (**23**). The resulting alcohols were hydrolyzed and dehydrated with HCl in a single step to afford the corresponding unsaturated ketones **26b**−**d**, **f**. Similarly, the intermediate ketone **26e** was prepared starting from the organolithium derivative of 1,3-dimethoxybenzene (**24**). The intermediates **26b**−**e** were not isolated and were reduced with H_2 and 10% Pd on activated carbon to obtain key-compounds **28b**−**e**.[24] Intermediate ketone **28a** was commercially available, whereas attempts to prepare compound **28f**, by reduction of corresponding unsaturated ketone **26f**, failed.

Intermediate ketone **28f** was then obtained through a different synthetic pathway,[25] still starting from Grignard's reagent of compound **22f**. In the presence of a catalytic amount of $FeCl_3$ and TMEDA (*N*,*N*,*N*,*N*-tetramethylethylendiamine), 2-bromomagnesium-*m*-xylene was cross-coupled with 8-bromo-1,4-dioxa-spiro[4,5]-decane (**25**), which was prepared from the ketal **23** by reduction to the corresponding alcohol with $NaBH_4$ and subsequent bromination with CBr_4.[26] The resulting intermediate **27** was deprotected to the corresponding ketone **28f**. The intermediates **28a**−**f** were subsequently condensed with 1-(2-pyridinyl)piperazine to the corresponding enamines, that were reduced in situ with $NaCNBH_3$ and $ZnCl_2$ to give the final compounds *cis*/*trans*-**29**−**34** as mixtures of geometric isomers. Column chromatography purification of the mixture provided the separation of each *cis*- from the *trans*-stereoisomers. Compound **34** was obtained in a very small amount and only the *cis*-isomer was isolated and identified.

For the synthesis of compound **35**, the intermediate **26f** was isolated and condensed with 1-(2-pyridinyl)piperazine to the

Scheme 1[a]



[a] Reagents: (A) Mg turnings; (B) HCl or H_2SO_4; (C) TMEDA, $FeCl_3$; (D) *n*-BuLi; (E) H_2, Pd/C 10%; (F) 1-(2-pyridinyl)piperazine, $ZnCl_2$, $NaCNBH_3$.

corresponding enamine, which was reduced in situ with $NaCNBH_3$ to give the desired compound as a racemic mixture. All final 1-(2-pyridinyl)piperazine derivatives were converted to the corresponding hydrochloride salts with gaseous HCl in the usual way. Their physical properties are listed in Table 2, along with the calculated values of the logarithm of the distribution coefficient (ClogD) at pH 7.4 for the corresponding free bases.[27]

Biology

Receptor Binding Studies. The already reported compounds **15**, **16**, *cis*-**18**, *cis*-**19**, *trans*-**19**, *cis*-**21** and *trans*-**21** were assayed as free bases, and *cis*-**14** as hydrogen oxalate salt. The compounds **13**, *cis*-**17** and *cis*-**20**, *trans*-**20** and the novel target compounds **29**−**35** were assayed as hydrochloride salts. All the compounds were evaluated for in vitro affinity at EBP site and at σ_1 and σ_2 receptors by radioreceptor binding assays. The cyclohexylpiperazine **36** (PB 28, Chart 3, structure **11** with 5-OCH_3 and R = cyclohexyl)[28] was tested for radioligand

Table 1. Binding Affinity Data for Preliminarily Screened Compounds

	$K_i \pm$ SEM (nM)					
compound	5-HT_{1A}[a]	D_2[a]	α_1[a]	EBP	σ_1	σ_2
13	676 ± 28	>850	585 ± 60	32.1 ± 4.2	358 ± 7	1250 ± 20
cis-**14**	373 ± 18	783 ± 32	>850	23.5 ± 2.7	7.71 ± 1.79	
15	257 ± 17	2190 ± 300	5160 ± 130	460[b]		
16	536 ± 24	1220 ± 120	512 ± 16	2640[b]	220 ± 80	
cis-**17**	74 ± 8	1310 ± 150	5540 ± 250	16.7 ± 2.2	240[b]	
cis-**18**	183 ± 22	2380 ± 160	2250 ± 160	1710[b]		
cis-**19**	480 ± 15	2120 ± 130	712 ± 35	8.15 ± 1.96	[c]	[c]
cis-**20**	815 ± 20	>850	783 ± 32	45.4 ± 11.5	[c]	[c]
cis-**21**	94 ± 8	787 ± 43	786 ± 29	32.8 ± 0.2	[c]	[c]

[a] Data already reported (ref 23). [b] Result from a unique experiment. [c] See Table 3.

Biology

Receptor Binding Studies. The already reported compounds **15**, **16**, *cis*-**18**, *cis*-**19**, *trans*-**19**, *cis*-**21** and *trans*-**21** were assayed as free bases, and *cis*-**14** as hydrogen oxalate salt. The compounds **13**, *cis*-**17** and *cis*-**20**, *trans*-**20** and the novel target compounds **29**−**35** were assayed as hydrochloride salts. All the compounds were evaluated for in vitro affinity at EBP site and at σ_1 and σ_2 receptors by radioreceptor binding assays. The cyclohexylpiperazine **36** (PB 28, Chart 3, structure **11** with 5-OCH_3 and R = cyclohexyl)[28] was tested for radioligand

Table 2. Physical Properties of Novel Compounds

compound	formula[a]	mp, °C[b]	ClogD[c]
cis-**29**	$C_{21}H_{27}N_3 \cdot 2HCl \cdot {}^3/_4H_2O$	268 (dec)	3.13
trans-**29**	$C_{21}H_{27}N_3 \cdot 2HCl$	248 (dec)	3.13
cis-**30**	$C_{22}H_{29}N_3 \cdot 2HCl \cdot H_2O$	285 (dec)	3.58
trans-**30**	$C_{22}H_{29}N_3 \cdot 2HCl \cdot {}^1/_2H_2O$	288 (dec)	3.58
cis-**31**	$C_{21}H_{26}N_3Cl \cdot 2HCl \cdot {}^5/_4H_2O$	278 (dec)	3.73
trans-**31**	$C_{21}H_{26}N_3Cl \cdot 2HCl \cdot {}^5/_4H_2O$	272 (dec)	3.73
cis-**32**	$C_{21}H_{25}N_3F_2 \cdot 2HCl \cdot {}^1/_2H_2O$	248 (dec)	3.29
trans-**32**	$C_{21}H_{25}N_3F_2 \cdot 2HCl \cdot {}^1/_2H_2O$	280 (dec)	3.29
cis-**33**	$C_{23}H_{31}N_3O_2 \cdot 2HCl \cdot H_2O$	194−196	2.84
trans-**33**	$C_{23}H_{31}N_3O_2 \cdot 2HCl \cdot 2H_2O$	296 (dec)	2.84
cis-**34**	$C_{23}H_{31}N_3 \cdot 2HCl \cdot {}^1/_2H_2O$	ND[d]	4.04
35	$C_{23}H_{29}N_3 \cdot 2HCl \cdot {}^1/_2H_2O$	285 (dec)	3.89

[a] Elemental analyses were within ±0.4% of the theoretical values for the formulas given. [b] Recrystallized from MeOH/Et_2O. [c] Referred to the corresponding free bases at pH 7.4; isomeric compounds **19**−**21** displayed a ClogD value of 3.04. [d] Not determined, because it was obtained in a very small amount.

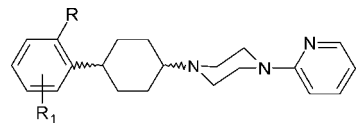
binding assay at EBP site, before being used as reference compound in the antiproliferative assay. The specific radioligands and tissue sources were, respectively, (a) EBP site, (±)-[^{3}H]-**1**, guinea-pig liver membranes; (b) σ_1 receptor, (+)-[^{3}H]-pentazocine ((+)-[2*S*-(2α,6α,11*R*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocine-8-ol), guinea-pig brain membranes without cerebellum; (c) σ_2 receptor, [^{3}H]-DTG (1,3-di-2-tolylguanidine) in the presence of 1 μM (+)-pentazocine to mask σ_1 receptors, rat liver membranes. The following compounds were used to define the specific binding reported in parentheses: (a) (±)-ifenprodil (67−85%), (b) (+)-pentazocine (75−84%), (c) DTG (82−93%). Concentrations required to inhibit 50% of radioligand specific binding (IC_{50}) were determined by using six to nine different concentrations of the drug studied in two or three experiments with samples in duplicate. Scatchard parameters (K_d and B_{max}) and apparent inhibition constants (K_i) values were determined by nonlinear curve fitting, using the Prism v. 3.0, GraphPad software.[29]

Antiproliferative and Cytotoxic Assays. The functional biochemical assays were carried out on androgen-unsensitive PC-3 cell line of human prostate epithelial carcinoma, where the expression of EBP site and σ_1 receptor had been previously reported.[15] Among the EBP ligands newly synthesized, five compounds with different binding profile were selected. The compounds *cis*-**19**, *cis*-**30** and *cis*-**33** were chosen as the highest-affinity and selective EBP ligands, along with EBP relative to σ_1 selective ligand *cis*-**32** with moderate EBP affinity and *trans*-**20** as a nonselective one. Furthermore, the following positive reference compounds were tested: EBP-inhibitor ifenprodil, σ_2 agonist **36**, σ_1 antagonist *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine (**37**, NE 100).[30] To complete the assay panel, the following agents were also tested: σ_1 agonist (+)-pentazocine, σ_2 antagonist *N*-(2-phenylethyl)piperidine (**38**, AC 927) and mixed σ_1/σ_2 agonist DTG. All selected compounds were tested to evaluate their possible EBP-mediated antiproliferative effect at 48 h, and cytotoxic effect at 24 h in PC-3 cell line. The maximum cytotoxic effect was assessed when the Fluorescence Units from LDH (Lactate dehydrogenase) release by treated cells in medium were the same as from total LDH measured from total lysis of cells in untreated control. The EC_{50} values were obtained from nonlinear iterative curve fitting by Prism v. 3.0, GraphPad software.

Results and Discussion

Radioligand Binding and EBP SAfiR. For a preliminary screening, the lowest-affinity ligands at serotonin 5-HT_{1A}, dopamine D_2 and adrenergic α_1 receptors were selected among various arylpiperazines previously tested (Chart 4).[23] The

Table 3. Binding Affinities and Selectivities



			$K_i \pm$ SEM (nM)			K_i ratio	
compound	R	R_1	EBP	σ_1	σ_2	σ_1/EBP	σ_2/EBP
cis-**19**	OCH_3	H	8.15 ± 1.96	369 ± 116	242 ± 61	45	30
trans-**19**	OCH_3	H	13.5 ± 2.6	2.86 ± 0.95	30.0 ± 8.8	0.2	2.2
cis-**20**	H	3-OCH_3	45.4 ± 11.5	178 ± 57	36.1 ± 9.0	4	0.8
trans-**20**	H	3-OCH_3	16.2 ± 2.0	24.8 ± 6.5	21.9 ± 2.1	1.5	1.3
cis-**21**	H	4-OCH_3	32.8 ± 0.2	194 ± 85	63.8 ± 21.9	6	2
trans-**21**	H	4-OCH_3	71.6 ± 15.1	291 ± 3	222 ± 19	4	3
cis-**29**	H	H	49.2 ± 6.8	802 ± 43	62.0 ± 5.3	16	1.3
trans-**29**	H	H	16.8 ± 4.2	104 ± 10	26.9 ± 7.2	6	1.6
cis-**30**	CH_3	H	5.00 ± 1.50	1000 ± 20	71.0 ± 4.0	200	14
trans-**30**	CH_3	H	9.04 ± 1.56	30.7 ± 7.1	29.6 ± 4.4	3.4	3.3
cis-**31**	Cl	H	85.1 ± 25.9	1090 ± 50	150 ± 43	13	1.8
trans-**31**	Cl	H	7.21 ± 1.71	190 ± 67	29.1 ± 10.4	26	4
cis-**32**	F	6-F	140 ± 2	5170 ± 280	440 ± 39	37	3
trans-**32**	F	6-F	106 ± 6	190 ± 6	138 ± 54	1.8	1.2
cis-**33**	OCH_3	6-OCH_3	4.95 ± 1.73	>6000	687 ± 66	>1210	138
trans-**33**	OCH_3	6-OCH_3	7.00 ± 1.04	215 ± 10	25.0 ± 5.4	31	3.6
cis-**34**	CH_3	6-CH_3	33.1 ± 2.1	6.32 ± 1.07	60.2 ± 2.2	0.2	1.8
35[a]	CH_3	6-CH_3	46.7 ± 15.0	177 ± 15	113 ± 27	3.8	2.4
36			8.38 ± 0.81	13.6 ± 1.9[b]	0.34 ± 0.02[b]	1.6	0.04
37[c]			14.6 ± 4.1	1.03 ± 0.14	212 ± 24	0.07	14
38			595[d]	309 ± 15[e]	194 ± 23[e]	0.5	0.3
(+)-pentazocine			>10^4	2.41 ± 0.37	1860 ± 40[f]	<$2 \cdot 10^{-3}$	<2
DTG			7660 ± 790	88.5 ± 9.0[f]	31.1 ± 2.5	0.01	$4 \cdot 10^{-3}$
(±)-ifenprodil			16.4 ± 1.5	2 ± 0.2[g]	2.3[h]	0.12	0.14

[a] Racemic cyclohexyl-3-ene derivative (see formula in Scheme 1). [b] From ref 31. [c] Binding data from ref 19. [d] Result from a unique experiment. [e] From ref 28. [f] From ref 30. [g] From ref 18. [h] From ref 32.

binding data for these compounds are reported in Table 1 and are expressed as K_i values. As for EBP affinity, the results were encouraging only for compound *cis*-**19** (K_i = 8.15 nM). The compounds **13**, *cis*-**14**, *cis*-**17**, *cis*-**20** and *cis*-**21** displayed fairly lower EBP affinity, with K_i values ranging from 16.7 to 45.4 nM, whereas compounds **15**, **16** and *cis*-**18** displayed poor EBP affinity and were not further investigated. Some of these compounds were assayed in σ-receptors binding and compound *cis*-**19** demonstrated good selectivity relative to either σ_1 or σ_2 receptors. Even though they could be not selective, *trans*-stereoisomers of isomeric compounds *cis*-**19**−**21** were tested too, in order to complete SAfiR studies.

The results from binding assays (K_i values) at EBP site, σ_1 and σ_2 receptors are shown in Table 3 for compounds *cis*/*trans*-**19**−**33**, *cis*-**34** and **35**. The affinities of compounds **19**−**35** for the EBP site were not the highest among the affinities of known EBP ligands, but they were higher than or comparable to the corresponding σ-receptor affinities. Only compounds *trans*-**19** and *cis*-**34** displayed low K_i values in σ_1 receptor binding. As for EBP affinity, the K_i values ranged from 4.95 nM (compound *cis*-**33**) to 140 nM (compound *cis*-**32**). The highest EBP affinities were reached by cis-isomers *cis*-**33**, *cis*-**30** and *cis*-**19** (K_i = 4.95, 5.00, and 8.15 nM, respectively) and by *trans*-isomers *trans*-**33**, *trans*-**31** and *trans*-**30** (K_i = 7.00, 7.21, and 9.04 nM, respectively). Nevertheless, the 2,6-dimethoxy derivative *cis*-**33** displayed a more interesting selectivity profile compared to the corresponding *trans*-**33**. The same comment can be done for 2-methyl derivative *cis*-**30** compared to compound *trans*-**30** and for 2-methoxycompound *cis*-**19** compared to *trans*-**19**. Conversely, 2-chloroderivative *trans*-**31** was a better EBP ligand than the corresponding *cis*-**31** but only slightly more selective. The compound *cis*-**33** was the most selective EBP ligand within this set of compounds, >1210-fold relative to σ_1 receptor and 138-fold relative to σ_2 receptor.

As shown in Table 2, the ClogD values were included in a rather narrow range, covering about one log unit (2.84−4.04). Apparently, no evident relationship was proven between EBP affinity and ClogD. High-affinity EBP ligands *cis*-**33**, *cis*-**30** and *trans*-**31** (K_i = 4.95−7.21 nM) had ClogD ranging from 2.84 to 3.73. The lowest ClogD values were reached by the highest-affinity 2,6-dimethoxylated EBP ligands *cis*-**33** and *trans*-**33**. Conversely, the isomeric 2-methoxy derivatives **19**−**21** with the same ClogD = 3.04 displayed EBP affinities in a wide range of K_i values (8.15−71.6 nM).

Generally, not great differences in EBP affinities were noted between cis-compounds and trans-compounds. Moreover, the presence and the position of methoxyl groups on the phenyl ring, moderately affected the EBP affinity. As for monomethoxy derivatives, a small difference occurred between K_i values of the 2-methoxy isomer *cis*-**19** (K_i = 8.15 nM) and 3-methoxy isomer *cis*-**20** (K_i = 45.4 nM) or 4-methoxy isomer *cis*-**21** (K_i = 32.8 nM), or unsubstituted compound *cis*-**29**, excluding a significant influence of the electronic effect of methoxyl group. The lowest K_i value (4.95 nM) occurred for 2,6-dimethoxy derivative *cis*-**33**, evidencing the main role of steric effects in the ortho position. Moreover, 2,6-dimethoxy derivative *cis*-**33** presented an EBP affinity only equipotent to 2-monomethoxy derivative *cis*-**19**, but the former displayed lower σ_1 and σ_2 receptor affinities, resulting in a much better selectivity, particularly relative to σ_1 receptor.

For the cis-compounds the selectivity was high when the 2-position is occupied by a methoxyl or a methyl group; 2-chloro or 2-fluoro substituent were detrimental for EBP affinity and consequently for selectivity. Analogous considerations could be done for corresponding trans-isomers. When the structure is closer to planarity, as in the cyclohexenyl compound **35**, both EBP affinity and selectivity dropped. As for electronic effects, the K_i value of 2-chloro derivative *cis*-**31** (K_i = 85.1 nM) was 1 order of magnitude greater than that of 2-methoxy derivative *cis*-**19** (K_i = 8.15 nM). This was not true for the corresponding stereoisomers *trans*-**31** (K_i = 7.21 nM) and *trans*-**19** (K_i = 13.5 nM). Consistently, in the series of cis-isomers the electron-donor effect was beneficial as for methyl-substituted *cis*-**30** (K_i = 5.00 nM). The same effect was indifferent for the stereoisomers *trans*-**19**, *trans*-**31** and *trans*-**30**, which displayed similar EBP affinities. It is possible that the trans-compounds enjoy of a more conformational freedom, whereas cis-compounds are more constrained. The highest K_i value (K_i = 140 nM) for 2,6-difluoro derivative *cis*-**32** demonstrated the negative influence of small electron-withdrawing substituents, suggesting that electronic effects were added to the steric ones. This was confirmed by compound *trans*-**32**, which demonstrated the worst EBP affinity in the set of trans-compounds. The 2,6-dimethylderivative *cis*-**34** showed an intermediate value (K_i = 33.1 nM) between those of dimethoxy compound *cis*-**33** and difluoro compound *cis*-**32**.

Table 4. Antiproliferative Effect Measured as Inhibition of PC-3 Cancer Cell[a] Proliferation

compound	EC_{50} ± SEM[b] (μM)	compound	EC_{50} ± SEM[b] (μM)
cis-**19**	19.3 ± 8.7	**37**	47.0 ± 12.8
trans-**20**	23.0 ± 2.5	**38**	(30%)[c]
cis-**30**	38.0 ± 6.0	(±)-ifenprodil	25.8 ± 3.79
cis-**32**	37.3 ± 3.3	(+)-pentazocine	86.9 ± 16.7
cis-**33**	12.9 ± 2.9	DTG	(27%)[c]
36	37.7 ± 1.3		

[a] Human prostate carcinoma epithelial cell lines. [b] Mean of $n \geq 2$ separate experiments. [c] Percent inhibition at 100 μM.

***σ* SafiR.** All compounds were selected as potentially low-affinity σ ligands; nevertheless, compounds *trans*-**19** (K_i = 2.86 nM) and *cis*-**34** (K_i = 6.32 nM) displayed preferentially σ_1 receptor affinity and a low selectivity. Several compounds, mainly in the trans-isomers series displayed moderate σ_2 affinity. The lowest σ_1 affinities were recorded for compounds *cis*-**33**, *cis*-**32**, *cis*-**31** and *cis*-**30** ($K_i \geq$ 1000 nM), all belonging to the cis-isomers series. Within this series, the affinity for the σ_1 subtype dramatically dropped when the bulky group was in the 2-position, whereas the affinity for the EBP site was unchanged leading to higher EBP-site selectivity relative to σ_1 receptor. The highest K_i (>6000 nM) belonged to the best EBP ligand *cis*-**33**. Therefore, compound *cis*-**33** was the most selective high-affinity EBP ligand. In the trans-isomers series, the 2-substitution only slightly affected the σ_1 receptor affinity, leading to lower EBP relative to σ_1 selectivity. This same trend was also observed for the affinities toward the σ_2 receptor, although at a lower extent, with a higher decrease in the affinity within the cis-isomers series rather than in the trans-isomers series. These findings were in accordance with the virtual pharmacophore models of EBP and σ_1 receptor derived recently, where σ_1 receptor model preferentially bound linear ligands, as these new trans-compounds.[18]

Functional Assays and SAR. The results expressed as EC_{50} values were reported in Table 4. In the human prostate cancer PC-3 cell line, the σ/EBP receptor ligand **2** showed to exert its antiproliferative activity through the EBP inhibition with a nanomolar efficacy. σ_1 Subtype receptor was demonstrated to be not responsible for such effect, but interaction of **2** with the σ_2 subtype was hypothesized. In fact, inhibition of sterol isomerase activity alone could not fully account for the antiproliferative effect exerted by **2**: neither direct correlation

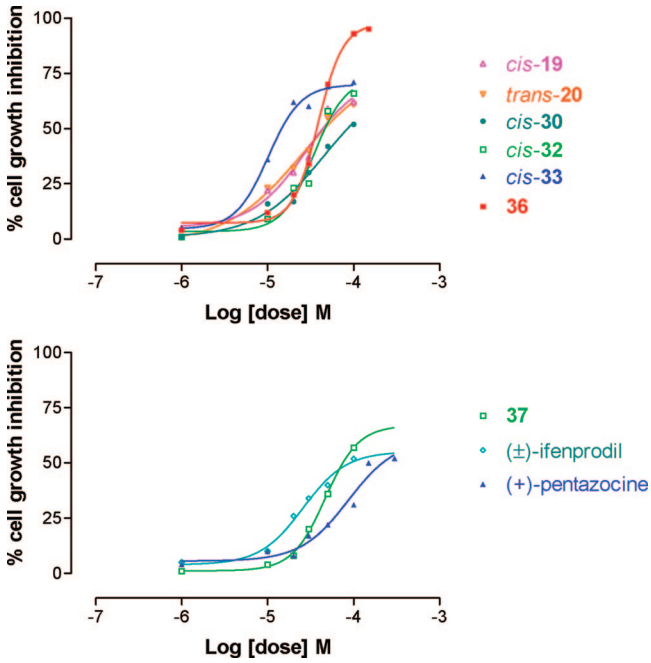


Figure 1. Antiproliferative effect of compounds tested in androgen-unsensitive PC-3 cell line of human prostate cancer. Incubation was carried out for 48 h in the presence (1−100 μM) and absence of tested compound (ref 15). For compound **36** and (+)-pentazocine, the concentration ranges were 1−150 and 1−300 μM, respectively.

between cellular EBP content and effect was found, nor the antiproliferative effect was completely reversed by the addition of cholesterol to the medium.[16] Thus, it appeared important to define the role of the σ_2 receptor in mediating the antiproliferative action in such cell lines. First, we recognized the σ_2 receptor content in PC-3 cell line and its density was found comparable to that of EBP sites. To understand the importance of the three binding sites in determining the antiproliferative effect, the following compounds were selected: *cis*-**33** and *cis*-**19** as selective EBP ligands almost devoid of σ receptors affinities, the former being the most selective and highest-affinity EBP ligand; *trans*-**20** for displaying equal and moderate affinity and *cis*-**32** for displaying low affinity toward the three sites; *cis*-**30** for being high affinity toward the EBP site and only slightly selective toward the σ_2 subtype. With the purpose to correlate the antiproliferative effect also with the σ receptor content, ifenprodil and some σ agents were evaluated. Almost all the tested compounds displayed a certain antiproliferative activity. Among the reference compounds, the σ_2 agonist **36** was the only one reaching the maximum inhibition of proliferation. Such result can be explained with the activity of compound **36** both on σ_2 receptor and EBP site. Compound *cis*-**33** did not reach the maximum inhibition (70%), although it was more potent (EC_{50} = 12.9 μM) than **36** (Figure 1), maybe for its lack of affinity to the σ_2 receptor subtype (K_i = 687 nM). Lower efficacy and potency were shown by compound *cis*-**19** in comparison to *cis*-**33**, as expected by the slightly lower EBP affinity of *cis*-**19** along with its low affinity for the σ_2 receptor. Although *cis*-**30** displayed rather higher EBP affinity than *cis*-**32**, this latter surprisingly was more potent and effective than *cis*-**30**. This result may be explained with a possible *cis*-**30** antagonist activity at σ_2 receptor. The other tested compounds did not reach 70% efficacy at 100 μM (Figure 1). Among the reference compounds, ifenprodil presented the best potency value (EC_{50} = 25.8 μM). Its efficacy was a little over 50% as for σ_1 receptor agents (+)-pentazocine and **37**, but these latter were less potent than ifenprodil. As expected, σ_1 antagonist **37** had a higher potency and efficacy than (+)-pentazocine. DTG and compound **38** were practically inactive in this assay. All tested compounds displayed poor cytotoxic activity (<10% at 100 μM), while only compound **36** had a moderately cytotoxic activity (EC_{50} = 64.2 μM).

The ClogD values for the tested compounds increased from 2.84 for the most active compound *cis*-**33** to 3.58 for the less active *cis*-**30**, displaying a linear relationship between antiproliferative activity and ClogD. In this respect, in the most active compounds *cis*-**33**, *cis*-**19** and *trans*-**20**, the methoxyl groups may contribute to the activity also by decreasing ClogD. The reference compound **36** had a ClogD = 3.75.

Conclusions

A new class of selective EBP ligands was found by opening the tetralin moiety and constraining the intermediate chain of (tetralinalkyl)arylpiperazines in the arylcyclohexyl-1-(2-pyridyl)piperazine derivatives. The electronic and steric effects were investigated in a series of semirigid 2- and 2,6-disubstituted *cis*- and *trans*-phenylcyclohexyl derivatives. Several compounds, particularly in the cis series, displayed nanomolar affinity and good selectivities relative to σ_1 and σ_2 receptors. The 2,6-dimethoxy derivative *cis*-**33** showed the highest EBP affinity and the best selectivities. In an antiproliferative assay on PC-3 cell line, *cis*-**33** demonstrated the highest potency but not the best efficacy that was reached by σ_2 agonist **36**. Compound *cis*-**33** appeared as a valuable pharmacological tool for investigating enzyme activity in the last steps of cholesterol biosynthesis. Its partial antiproliferative activity has to be further investigated to define the role of σ_2 receptor activation in this assay. Nevertheless *cis*-**33** may represent a starting point for a new approach to anticancer treatment.

Experimental Section

Chemistry. Column chromatography was performed with 1:30 ICN silica gel 60 Å (63−200 or 15−40 μm, respectively) as the stationary phase. Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. Elemental analyses (C, H, N) were performed on a Eurovector Euro EA 3000 analyzer; the analytical results were within ±0.4% of the theoretical values. ^{1}H NMR spectra were recorded at 300 MHz on a Mercury Varian spectrometer using $CDCl_3$ as solvent unless otherwise reported. The chemical shift values were reported in ppm (δ). Recording of mass spectra was done on an Agilent 6890−5973 MSD gas chromatograph/mass spectrometer and on an Agilent 1100 series LC-MSD trap system VL; only significant *m*/*z* peaks, with their percentage of relative intensity in parentheses, are reported. Chemicals were from Aldrich and Across and were used without any further purification.

General Procedure To Obtain Final 4-(2-Pyridinyl)piperazine Derivatives *cis*/*trans*-29−33, *cis*-34, and 35. In a typical reaction, one intermediate (1.0 mmol) among the 4-phenylcyclohexanone derivatives **28a**−**f** and 4-(2,6-dimethylphenyl)-3-cyclohexen-1-one (**26f**) were reacted with the 1-(2-pyridinyl)piperazine (1.0 mmol), zinc(II) chloride (0.58 mmol) and $NaCNBH_3$ (1.1 mmol) in 2-propanol (20 mL). The mixture was stirred for 48 h at room temperature. Then, the reaction mixture was evaporated to dryness and the residue was diluted with 2 N NaOH (20 mL) and extracted with AcOEt (3 × 20 mL). The organic layers were washed with brine (20 mL), dried (Na_2SO_4) and then concentrated under reduced pressure to give a crude residue. Purification by column chromatography afforded the separated cis- and trans-isomers of final compounds. Consistently, the cis-stereoisomers were eluted before their trans counterparts. Attribution of cis/trans geometry was done on the basis of ^{1}H NMR chemical shifts and coupling

constants for NCH and CHAr in the cyclohexyl ring, assisted also by NOESY-NMR.

***cis*-4-(4-Phenylcyclohexyl)-1-(2-pyridinyl)piperazine (*cis*-29)** was obtained as white crystals in 35% yield with CH_2Cl_2/AcOEt (4:6) as eluent: ^{1}H NMR δ 1.50−1.72 (m, 4H, cyclohexylic), 1.94−2.18 (m, 4H, cyclohexylic), 2.27−2.37 (m, 1H, NCH), 2.56−2.77 [m, 5H, benzyl CH and CHN(CH_2)$_2$], 3.48−3.62 [m, 4H, (CH$_2$)$_2$NPy], 6.58−6.68 (m, 2H, Py), 7.14−7.21 (m, 1H, aromatic), 7.25−7.32 (m, 4H, aromatic), 7.44−7.51 (m, 1H, Py), 8.17−8.22 (m, 1H, Py N=CH); GC−MS *m/z* 322 (M^+ + 1, 6), 321 (M^+, 21), 227 (42), 107 (100). Anal. ($C_{21}H_{27}N_3 \cdot 2HCl \cdot {}^3/_4H_2O$) C, H, N.

***trans*-4-(4-Phenylcyclohexyl)-1-(2-pyridinyl)piperazine (*trans*-29)** was obtained as white crystals in 30% yield with CH_2Cl_2/AcOEt (4:6) as eluent: ^{1}H NMR δ 1.40−1.62 (m, 4H, cyclohexylic), 1.95−2.12 (m, 4H, cyclohexylic), 2.40−2.56 (m, 2H, benzyl CH and NCH), 2.71−2.82 [m, 4H, CHN(CH_2)$_2$], 3.52−3.64 [m, 4H, (CH$_2$)$_2$NPy], 6.58−6.68 (m, 2H, Py), 7.15−7.32 (m, 5H, aromatic), 7.44−7.52 (m, 1H, Py), 8.16−8.22 (m, 1H, Py N=CH); GC−MS *m/z* 322 (M^+ + 1, 4), 321 (M^+, 16), 227 (31), 107 (100). Anal. ($C_{21}H_{27}N_3 \cdot 2HCl$) C, H, N.

***cis*-4-[4-(2-Methylphenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*cis*-30)** was obtained as white crystals in 42% yield with CH_2Cl_2/AcOEt (1:1) as eluent: ^{1}H NMR δ 1.47−1.62 (m, 4H, cyclohexylic), 1.84−2.00 (m, 2H, cyclohexylic), 2.08−2.19 (m, 2H, cyclohexylic), 2.27−2.34 (m, 1H, NCH), 2.35 (s, 3H, CH$_3$), 2.58−2.66 [m, 4H, CHN(CH_2)$_2$], 2.85 (tt, 1H, J = 11.4 Hz, J' = J'' = 3.58 Hz, ax benzyl CH), 3.53−3.60 [m, 4H, (CH$_2$)$_2$NPy], 6.58−6.70 (m, 2H, Py), 7.04−7.22 (m, 3H, aromatic), 7.30 (d, 1H, J = 6.9 Hz, aromatic), 7.44−7.53 (m, 1H, Py), 8.17−8.24 (m, 1H, Py N=CH); GC−MS *m/z* 336 (M^+ + 1, 5), 335 (M^+, 19), 216 (64), 107 (100). Anal. ($C_{22}H_{29}N_3 \cdot 2HCl \cdot H_2O$) C, H, N.

***trans*-4-[4-(2-Methylphenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*trans*-30)** was obtained as white crystals in 36% yield with CH_2Cl_2/AcOEt (4:6) as eluent: ^{1}H NMR δ 1.43−1.60 (m, 4H, cyclohexylic), 1.87−1.98 (m, 2H, cyclohexylic), 2.06−2.17 (m, 2H, cyclohexylic), 2.33 (s, 3H, CH$_3$), 2.45−2.58 (m, 1H, NCH), 2.64−2.87 [m, 5H, benzyl CH and CHN(CH_2)$_2$], 3.53−3.68 [m, 4H, (CH$_2$)$_2$NPy], 6.60−6.69 (m, 2H, Py), 7.05−7.22 (m, 4H, aromatic), 7.44−7.53 (m, 1H, Py), 8.17−8.23 (m, 1H, Py N=CH); GC−MS *m/z* 336 (M^+ + 1, 4), 335 (M^+, 15), 216 (49), 107 (100). Anal. ($C_{22}H_{29}N_3 \cdot 2HCl \cdot {}^1/_2H_2O$) C, H, N.

***cis*-4-[4-(2-Chlorophenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*cis*-31)** was obtained as white crystals in 20% yield with CH_2Cl_2/AcOEt (1:1) as eluent: ^{1}H NMR δ 1.52−1.68 (m, 4H, cyclohexylic), 1.70−1.97 (m, 2H, cyclohexylic), 2.07−2.18 (m, 2H, cyclohexylic), 2.25−2.36 (m, 1H, NCH), 2.54−2.70 [m, 4H, CHN(CH_2)$_2$], 3.08−3.22 (br t, 1H, J = 11.4 Hz, benzyl CH), 3.48−3.66 [m, 4H, (CH$_2$)$_2$NPy], 6.60−6.69 (m, 2H, Py), 7.10 (dt, 1H, J = 7.6 Hz, J' = 1.6 Hz, aromatic), 7.19−7.24 (br t, 1H, aromatic), 7.33 (dd, 2H, J = 7.7 Hz, J' = 1.4 Hz, aromatic), 7.45−7.52 (m, 1H, Py), 8.18−8.21 (m, 1H, Py N=CH); GC−MS *m/z* 357 (M^+ + 2, 4), 356 (M^+ + 1, 3), 355 (M^+, 11), 236 (44), 107 (100). Anal. ($C_{21}H_{26}N_3Cl \cdot 2HCl \cdot {}^5/_4H_2O$) C, H, N.

***trans*-4-[4-(2-Chlorophenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*trans*-31)** was obtained as white crystals in 20% yield with CH_2Cl_2/AcOEt (1:1) as eluent: ^{1}H NMR δ 1.40−1.70 (m, 4H, cyclohexylic), 1.98−2.20 (m, 4H, cyclohexylic), 2.53−2.66 (m, 1H, NCH), 2.75−2.90 [m, 4H, CHN(CH_2)$_2$], 2.93−3.07 (br t, 1H, J = 11.6 Hz, benzyl CH), 3.58−3.75 [m, 4H, (CH$_2$)$_2$NPy], 6.61−6.68 (m, 2H, Py), 7.09−7.15 (m, 1H, aromatic), 7.18−7.25 (m, 2H, aromatic), 7.34 (dd, 1H, J = 8.3 Hz, aromatic), 7.45−7.53 (m, 1H, Py), 8.18−8.21 (m, 1H, Py N=CH); GC−MS *m/z* 357 (M^+ + 2, 3), 356 (M^+ + 1, 3), 355 (M^+, 9), 236 (35), 107 (100). Anal. ($C_{21}H_{26}N_3Cl \cdot 2HCl \cdot {}^5/_4H_2O$) C, H, N.

***cis*-4-[4-(2,6-Difluorophenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*cis*-32)** was obtained as white crystals in 19% yield with CH_2Cl_2/AcOEt (4:6) as eluent: ^{1}H NMR δ 1.39−1.63 (m, 4H, cyclohexylic), 2.07−2.38 (m, 5H, cyclohexylic), 2.55−2.68 [m, 4H, CHN(CH_2)$_2$], 3.10 (br t, 1H, J = 12.1 Hz, benzyl CH), 3.48−3.65 [m, 4H, (CH$_2$)$_2$NPy], 6.58−6.70 (m, 2H, Py), 6.80 (t, 2H, J = 8.5 Hz, aromatic), 7.03−7.14 (m, 1H, aromatic), 7.44−7.52 (m, 1H, Py), 8.18−8.22 (m, 1H, Py N=CH); GC−MS *m/z* 358 (M^+ + 1, 5), 357 (M^+, 15), 238 (58), 107 (100). Anal. ($C_{21}H_{25}N_3F_2 \cdot 2HCl \cdot {}^1/_2H_2O$) C, H, N.

***trans*-4-[4-(2,6-Difluorophenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*trans*-32)** was obtained as white crystals in 17% yield with CH_2Cl_2/AcOEt (4:6) as eluent: ^{1}H NMR δ 1.34−1.52 (m, 2H, cyclohexylic), 1.80−2.10 (m, 6H, cyclohexylic), 2.48 (tt, 1H, J = 11.6 Hz, J' = 3.4 Hz, NCH), 2.68−2.77 [m, 4H, CHN(CH_2)$_2$], 2.90−3.02 (m, 1H, benzyl CH), 3.50−3.58 [m, 4H, (CH$_2$)$_2$NPy], 6.58−6.68 (m, 2H, Py), 6.81 (t, 2H, J = 8.5 Hz, aromatic), 7.05−7.15 (m, 1H, aromatic), 7.43−7.51 (m, 1H, Py), 8.17−8.21 (m, 1H, Py N=CH); GC−MS *m/z* 358 (M^+ + 1, 4), 357 (M^+, 15), 238 (43), 107 (100). Anal. ($C_{21}H_{25}N_3F_2 \cdot 2HCl \cdot {}^1/_2H_2O$) C, H, N.

***cis*-4-[4-(2,6-Dimethoxyphenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*cis*-33)** was obtained as white crystals in 43% yield with CH_2Cl_2/AcOEt 1:1 as eluent: ^{1}H NMR δ 1.18−1.31 (m, 1H, cyclohexylic), 1.42−1.56 (m, 2H, cyclohexylic), 1.58−1.67 (m, 1H, cyclohexylic), 2.02−2.14 (m, 2H, cyclohexylic), 2.25−2.30 (br m, 1H, NCH), 2.41−2.72 [m, 6H, 2H cyclohexylic and CHN(CH_2)$_2$], 3.32 (tt, 1H, J = 12.2 Hz, J' = 4.0 Hz, benzyl CH), 3.50−3.65 [m, 4H, (CH$_2$)$_2$NPy], 3.78 (s, 6H, 2 OCH$_3$), 6.53 (d, 2H, J = 8.3 Hz, aromatic), 6.58−6.66 (m, 1H, Py), 6.68 (d, 1H, J = 8.5 Hz, Py), 7.09 (t, 1H, J = 8.4 Hz, aromatic), 7.45−7.53 (m, 1H, Py), 8.18−8.23 (m, 1H, Py N=CH); GC−MS *m/z* 382 (M^+ + 1, 5), 381 (M^+, 20), 274 (70), 262 (100), 151 (81), 107 (92). Anal. ($C_{23}H_{31}N_3O_2 \cdot 2HCl \cdot H_2O$) C, H, N.

***trans*-4-[4-(2,6-Dimethoxyphenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*trans*-33)** was obtained as white crystals in 36% yield with CH_2Cl_2/AcOEt (4:6) as eluent: ^{1}H NMR δ 1.35−1.54 (m, 2H, cyclohexylic), 1.61−1.73 (m, 2H, cyclohexylic), 1.98−2.26 (m, 4H, cyclohexylic), 2.47−2.63 (m, 1H, NCH), 2.72−2.89 [m, 4H, CHN(CH_2)$_2$], 3.20 (tt, 1H, J = 12.1 Hz, J' = 3.5 Hz, benzyl CH), 3.54−3.70 [m, 4H, (CH_2)$_2$Py], 3.78 (s, 6H, OCH$_3$), 6.53 (d, 2H, J = 8.3 Hz, aromatic), 6.58−6.70 (m, 2H, Py), 7.10 (t, 1H, J = 8.3 Hz, aromatic), 7.44−7.53 (m, 1H, Py), 8.17−8.23 (m, 1H, Py N=CH); GC−MS *m/z* 382 (M^+ + 1, 5), 381 (M^+, 19), 274 (58), 262 (88), 151 (85), 107 (100). Anal. ($C_{23}H_{31}N_3O_2 \cdot 2HCl \cdot 2H_2O$) C, H, N.

***cis*-4-[4-(2,6-Dimethylyphenyl)cyclohexyl]-1-(2-pyridinyl)piperazine (*cis*-34)** was afforded by column chromatography in 36% yield with AcOEt as eluent. Only the cis-isomer was obtained in enough amount for analytical purposes; ^{1}H NMR δ 1.38−1.45 (m, 4H, cyclohexylic), 1.78−1.82 (m, 2H, cyclohexylic), 1.90−2.18 (m, 2H, cyclohexylic), 2.20−2.52 (m, 7H, NCH and 2 CH$_3$), 2.68−2.80 [m, 4H, CHN(CH_2)$_2$], 2.85−3.02 (m, 1H, benzyl CH), 3.50−3.62 [m, 4H, (CH$_2$)$_2$NPy], 6.58−6.70 (m, 2H, Py), 6.82−7.00 (m, 3H, aromatic), 7.42−7.58 (m, 1H, Py), 8.17−8.22 (m, 1H, Py N=CH); GC−MS *m/z* 349 (M^+, 3), 230 (30), 107 (100). LC−MS on the hydrochloride salt (ESI$^+$) *m/z* 350 [M + H$^+$]; LC−MS−MS 350: 149, 121. Anal. C, H, N: not determined, due to the lack of product.

4-[1-(2,6-Dimethylphenyl)-3-cyclohexen-4-yl]-1-(2-pyridinyl)piperazine (35) was obtained as an oil in 76% yield with CH_2Cl_2/AcOEt (1:1) as eluent: ^{1}H NMR δ 1.43−1.78 (m, 2H, CHCH_2CH$_2$), 2.10−2.48 (mm, 5H, 2 allylic CH$_2$ and NCH), 2.19 (s, 3H, CH$_3$), 2.23 (s, 3H, CH$_3$), 2.64−2.86 [m, 4H, CHN(CH_2)$_2$], 3.48−3.70 [m, 4H, (CH$_2$)$_2$NPy], 5.38−5.47 (m, 1H, vinyl CH), 6.58−6.72 (m, 2H, Py), 6.98−7.08 (m, 3H, aromatic), 7.44−7.53 (m, 1H, Py), 8.17−8.23 (m, 1H, Py N=CH); GC−MS *m/z* 338 (M^+ + 1, 6), 337 (M^+, 21), 240 (31), 107 (100). Anal. ($C_{23}H_{29}N_3 \cdot 2HCl \cdot {}^1/_2H_2O$) C, H, N.

Biological Methods: Radioligand Binding Assays. All the procedures for the binding assays were previously described. $\Delta_8-\Delta_7$ SI (EBP) binding was carried out according to Moebius et al.[17] σ_1 and σ_2 receptor binding were carried out according to Matsumoto et al.[33] The radioligand (±)-[^{3}H]-**1** (83 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]-DTG (30 Ci/mmol) and (+)-[^{3}H]-pentazocine (34 Ci/mmol) were purchased from PerkinElmer Life Sciences

(Zavantem, Belgium). (±)-Ifenprodil and DTG were purchased from Tocris Cookson, Ltd., U.K. (+)-Pentazocine was obtained from Sigma-Aldrich-RBI srl (Milan, Italy). Male Dunkin guinea-pigs and Wistar Hannover rats (250−300 g) were from Harlan, Italy.

Cell Culture. The human prostate cancer PC-3 cell line was obtained from Interlab Cell Line Collection (ICLC, Genoa). PC-3 line was routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified incubator at 37 °C with a 5% CO_2 atmosphere.

Antiproliferative Assay. Determination of cell growth was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay at 48 h.[30,34] On day 1, 10 000 cells/well were plated in 96-well plates in a volume of 200 μL and on day 2, the various drugs alone or in combination were added. In all the experiments, the various drug-solvents (ethanol, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with drugs, 0.5 mg/mL MTT was added to each well, and after 3 h incubation at 37 °C, the supernatant was removed. The formazan crystals were solubilized using 100 μL of DMSO and the absorbance values at 570 and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences.

Cytotoxicity Assay. The assay was performed using the CytoTox-One kit from Promega Corp. (Madison, WI) as reported by Colabufo et al.[30] with minor modifications. Cell death was determined as release of LDH into the culture medium. The percentage of cytotoxicity was calculated relative to the LDH release from total lysis of cells in untreated control. It is assumed here that the drug-treated wells and the control wells contain the same total number of cells (dead plus alive cells) at the end of the treatment period. Therefore, the cytotoxic effect of tested compounds was unaffected by underestimation of cytotoxicity that should occur because of decreased total number of cells in the treated samples compared to the untreated control. Cells were seeded into 96-well plates for optical performance in the fluorescent cell-based assay in 100 μL of complete medium in the presence or absence of different concentrations of test compounds. The plate was incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C and then 100 μL of substrate mix in assay buffer was added. Ten microliters of lysis solution was added to untreated wells in order to estimate total LDH. Plates were kept protected from light for 10 min at room temperature, and then 50 μL of stop solution was added to all wells. The fluorescence was recorded using a LS55 Luminescence Spectrometer PerkinElmer with a 560 nm excitation wavelength and a 590 nm emission wavelength. Percent cytotoxicity was estimated as follows: 100 × (LDH in medium of treated cells − culture medium background)/(total LDH in untreated cells − culture medium background).

Supporting Information Available: Elemental analyses of the end products, description of the preparation and spectroscopy data for the intermediate compounds **26f**, **27** and **28b**−**f**. This material is available free of charge via the Internet at http://pubs.acs.org.

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