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Affinity of various ligands for $GABA_A$ receptors containing $\alpha_4\beta_3\gamma_2$, $\alpha_4\gamma_2$, or $\alpha_1\beta_3\gamma_2$ subunits

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Abstract

The potency of 30 benzodiazepine binding site ligands from 14 different structural classes for inhibition of [^{3}H]Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate) binding to human embryonic kidney (HEK) 293 cells transiently transfected with $\alpha_4\beta_3\gamma_{2S}$ or $\alpha_1\beta_3\gamma_{2S}$ subunits of $GABA_A$ receptors was investigated. Most of these compounds were unable to significantly inhibit [^{3}H]Ro 15-4513 binding to $\alpha_4\beta_3\gamma_{2S}$ receptors under conditions where they potently inhibited binding to $\alpha_1\beta_3\gamma_{2S}$ receptors. Nevertheless, compounds from four different structural classes were identified which exhibited a high affinity for $\alpha_4\beta_3\gamma_{2S}$ receptors. Variation of the structure of these compounds could lead to new ligands selectively interacting with $\alpha_4\beta_3\gamma_{2S}$ receptors. Compounds interacting with $\alpha_4\beta_3\gamma_{2S}$ receptors were also able to inhibit [^{3}H]Ro 15-4513 binding to receptors consisting of $\alpha_4\gamma_{2S}$ subunits with comparable potency. These results support the conclusion that the α subunit is a major determinant of the benzodiazepine binding site properties of $GABA_A$ receptors containing α and γ subunits.

Keywords: $GABA_A$ receptor subtype, recombinant, $\alpha4\beta3\gamma2S$, $\alpha4\gamma2S$, $\alpha1\beta3\gamma2S$; Benzodiazepine binding site property

1. Introduction

$GABA_A$ receptors are ligand-gated chloride ion channels that can be modulated by benzodiazepines, barbiturates, neurosteroids, anesthetics, and convulsants. A variety of evidence indicates that these compounds exert their action via distinct allosteric binding sites on these receptors (Sieghart, 1995). Especially the benzodiazepine binding site of $GABA_A$ receptors attracted a lot of interest since it is currently assumed that most of the pharmacologically and therapeutically relevant actions of benzodiazepines are elicited by an interaction with these binding sites (Haefely et al., 1985).

Recently, 6α, 4β, 4γ, one δ and 2ρ subunits of $GABA_A$ receptors have been cloned and sequenced from vertebrate brain (Burt and Kamatchi, 1991; Sieghart, 1995) and it has been demonstrated that five subunits seem to assemble to form functional $GABA_A$ receptors (Nayeem et al., 1994). Other studies have indicated that an α, a β and a γ subunit have to coassemble to form recombinant receptors exhibiting properties resembling those of native $GABA_A$ receptors. Depending on the subunit combination used for transfection of cell lines, recombinant $GABA_A$ receptors with different pharmacological and electrophysiological properties do arise (Burt and Kamatchi, 1991; Sieghart, 1995).

In two recent reports it has been demonstrated that $GABA_A$ receptors consisting of α_4, β_2, and γ_2 subunits exhibit unique benzodiazepine binding site properties (Wisden et al., 1991; Wieland and Lüddens, 1994). However, only a few compounds were investigated for their ability to interact with the benzodiazepine binding site of these receptors in this study. In order to extend the knowledge on the properties of α_4 subunit containing receptors, in the present study the affinity of 31 benzodiazepine binding site ligands for $GABA_A$ receptors consisting of α_4, β_3, and γ_{2S} subunits was determined. This subunit combination was used, since recent experiments in our laboratory have indicated that receptors containing these subunits actually do exist in the brain (Kern and Sieghart, 1994). Results obtained were compared with those from receptors consisting of α_1, β_3, and γ_{2S} subunits. In addition, the affinity of some of these compounds for receptors consisting of α_4 and γ_{2S} subunits was investigated.

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2. Materials and methods

2.1. Materials

[^{3}H]Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate, specific activity 20.9 Ci/mmol) was purchased from DuPont-New England Nuclear, Dreieich, Germany. Compounds (see Tables 1 and 2 and Figs. 1 and 2 for structures) were obtained from the following sources: diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one), midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-a][1,4]benzodiazepine, Ro 15-4513, Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate), bretazenil (*t*-butyl(s)-8-bromo-11,12,13,13a-tetrahydro-9-oxo-9*H*-imidazo[1,5-a]pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate) (Hoffmann La Roche, Basle, Switzerland); cloxazolam (10-chloro-11b-(2-chlorophenyl)-2,3,7,11b-tetrahydrooxazolo[3,2-d][1,4]benzodiazepin-6(5*H*)-one, oxazolam (10-chloro-2,3,7,11b-tetrahydro-2-methyl-11b-phenyloxazolo[3,2-d][1,4]benzodiazepin-6(5*H*)-one) (Sankyo, Tokyo, Japan); clotiazepam (5-(2-chlorophenyl)-7-ethyl-1,3-dihydro-1-methyl-2*H*-thieno[2,3-e][1,4]diazepin-2-one) (Troponwerke, Cologne, Germany); alprazolam (8-chloro-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine), triazolam (8-chloro-6-(2-chlorophenyl)-1-methyl-4*H*-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) (Upjohn, Kalamazoo, MI, USA); methyl (β-CCM)-, ethyl (β-CCE)-, propyl (β-CCP)-ester of β-carboline-3-carboxylate, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), FG 7142 (β-carboline-3-carboxy-methylamide) (Ferrosan, Soeborg, Denmark); abecarnil (isopropyl-6-benzyloxy-4-methoxymethyl-β-carboline-3-carboxylate) (Schering, Berlin, Germany); CGS 8216 (2-phenylpyrazolo[4,3-c]quinolin-3-one), CGS 9895 (2-(4-methoxyphenyl)-pyrazolo[4,3-c]quinolin-3-one), CGS 9896 (2-(4-chlorophenyl)-pyrazolo[4,3-c]quinolin-3-one), CGS 20625 (2-(4-methoxyphenyl)2,3,5,6,7,8,9,10-octahydrocyclohepta-(b)pyrazolo[4,3-d]pyridin-3-one) (Ciba Geigy, Summit, NJ, USA); Cl 218872 (3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo[4,3-b]pyridazine) (American

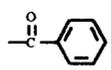
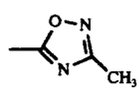
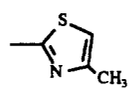
Table 1
Structure-affinity relationship of benzodiazepine binding site ligands for recombinant $\alpha_1\beta_3\gamma_{2S}$, $\alpha_4\beta_3\gamma_{2S}$, or $\alpha_4\gamma_{2S}$ $GABA_A$ receptors

No.	Compound	Structure	R_1	R_2	R_3	R_4	K_i (nM)		
							$\alpha_1\beta_3\gamma_{2S}$	$\alpha_4\beta_3\gamma_{2S}$	$\alpha_4\gamma_{2S}$
Imidazobenzodiazepines									
1	Ro 15-4513 (K_d)	I	$-N_3$	–	–	–	8.1 ± 0.6	5.5 ± 1.2	10.5 ± 5.5
2	Ro 15-1788	I	-F	–	–	–	2.9 ± 0.8	114 ± 33	265 ± 24
3	Bretazenil	II	–	–	–	–	0.45 ± 0.5	36 ± 10	22.5 ± 5
β-Carbolines									
4	DMCM	IX	$-COOCH_3$	$-C_2H_3$	$-OCH_3$	$-OCH_3$	7.5 ± 3.7	11.3 ± 1.7	20 ± 1
5	β-CCM	IX	$-COOCH_3$	-H	-H	-H	0.5 ± 0.1	640 ± 70	–
6	β-CCE	IX	$-COOC_2H_5$	-H	-H	-H	0.6 ± 0.3	693 ± 189	–
7	β-CCP	IX	$-COOC_3H_7$	-H	-H	-H	7.1 ± 0.2	922 ± 587	–
8	FG 7142	IX	$-CONHCH_3$	-H	-H	-H	160 ± 22	> 10000	–
9	Abecarnil	IX	$-COOCH(CH_3)_2$	$-CH_2OCH_3$	$-O-CH_2-C_6H_5$	-H	0.2 ± 0.1	1060 ± 230	–
Pyrazoloquinolines									
10	CGS 8216	XI	-H	–	–	–	0.11 ± 0.03	11.9 ± 2.8	–
11	CGS 9895	XI	$-OCH_3$	–	–	–	0.09 ± 0.04	4.0 ± 0.5	–
12	CGS 9896	XI	-Cl	–	–	–	0.26 ± 0.21	59 ± 10.4	–
Pyrazolopyridines									
13	CGS 20625	XII	–	–	–	–	0.15 ± 0.05	22.4 ± 5.3	77 ± 2
Triazolopyridazines									
14	Cl 218872	X	–	–	–	–	65 ± 10	> 10000	–
Imidazopyridines									
15	Zolpidem	XV	–	–	–	–	41 ± 12	> 10000	–

Membranes from HEK cells transfected with the subunit combination as indicated were incubated with 5 nM [^{3}H]Ro 15-4513 in the absence or presence of 100 μM Ro 15-1788 and various concentrations of the ligands investigated. The concentrations resulting in half maximal inhibition of specific [^{3}H]Ro 15-4513 binding (IC_{50}) were converted to K_i values by using the Cheng-Prusoff relationship (Cheng and Prusoff, 1973) and the respective K_d values for [^{3}H]Ro 15-4513 binding as given in Table 1. K_i values presented are mean values ± S.D. from 3–5 independent experiments performed in duplicate. 'Structure' refers to compound structures I–XVIII shown in Figs. 1 and 2. Values measured in $\alpha_4\gamma_{2S}$ transfected cells were significantly different from those in $\alpha_4\beta_3\gamma_{2S}$ transfected cells in Student's *t* test for Ro 15-1788 ($P = 0.005$), DMCM ($P = 0.01$), or CGS 20625 ($P = 0.005$), but were not significantly different for Ro 15-4513 ($P = 0.077$) or bretazenil ($P = 0.16$).

Cyanamide, Wayne, NJ, USA); zolpidem (*N,N*,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]-pyridine-3-acetamide) (Synthelabo Recherche, Bagneux, France); Ru 31 719 ((7-ethyl-5-methoxyimidazo[1,2-a]quinolin-2-yl) phenyl methanone), Ru 32 698 (6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)phenylmethanone, Ru 33 203 (5-(6-ethyl-7-methoxy-5-methylimidazo[1,2-a]-pyrimidin-2-yl)-3-methyl-[1,2,4]-oxadiazole), Ru 33 356 (2-(6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)-4-methyl-thiazole) (Roussel Uclaf, Romainville, France); PK 8165 (2-phenyl-4-(4-ethyl-piperidinyl)-quinoline), PK 9084 (2-phenyl-4-(3-ethyl-piperidinyl)-quinoline), PK 11195 (1-(2-chlorophenyl)-*N*-methyl-(1-methylpropyl)-3-isoquinoline carboxamide) (Pharmuka Laboratories, Gennevilliers, France); zopiclone (4-methyl-1-piperazinecarboxylic acid-6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5*H*-pyrrolo[3,4-b]-pyrazin-5-yl ester) (Rhone-Poulenc, Paris, France); clobazam (7-chloro-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione) (Hoechst, Frankfurt, Germany).

Table 2
[^{3}H]Ro 15-4513 binding to recombinant $GABA_A$ receptors consisting of $\alpha_1\beta_3\gamma_{2S}$ or $\alpha_4\beta_3\gamma_{2S}$ subunits in the presence of 10 μM concentrations of benzodiazepine binding site ligands

No.	Compound	Structure	R_1	R_2	[^{3}H]Ro 15-4513 binding (%)	
					$\alpha_1\beta_3\gamma_{2S}$	$\alpha_4\beta_3\gamma_{2S}$
1,4-Benzodiazepines						
16	Diazepam	III	–	–	1; 3	85; 93
17	Midazolam	IV	–	–	0; 2	94; 96
18	Alprazolam	V	-H	–	0; 4	75; 83
19	Triazolam	V	-Cl	–	5; 9	78; 82
20	Oxazolam	VII	$-CH_3$	-H	3; 7	100; 106
21	Cloxazolam	VII	-H	-Cl	52; 53	73; 104; 96
1,4-Thienodiazepines						
22	Clotiazepam	VI	–	–	1; 3	95; 92
1,5-Benzodiazepines						
23	Clobazam	VIII	–	–	7; 11	94; 99
Cyclopyrrolones						
24	Zopiclone	XVI	–	–	22; 25	92; 96
Imidazoquinolines						
25	Ru 31 719	XIII	–	–	10; 12	54; 64; 40
Imidazopyrimidines						
26	Ru 32 698	XIV		–	6; 10	62; 92
27	Ru 33 203	XIV		–	22; 26	96; 90
28	Ru 33 356	XIV		–	7; 11	71; 75
Quinolines						
29	PK 8165	XVII		–	11; 15	102; 107
30	PK 9084	XVII		–	6; 10	95; 98
Isoquinolines						
31	PK 11195	XVIII	–	–	53; 57	82; 99

Membranes from HEK cells transfected with the subunit combination as indicated were incubated with 5 nM [^{3}H]Ro 15-4513 in the absence or presence of 100 μM Ro 15-1788 and 10 μM of the compounds investigated. Data are given as % of the [^{3}H]Ro 15-4513 binding in the absence of the compounds investigated. Results are from two or three separate experiments performed in duplicate. 'Structure' refers to compound structures I–XVIII shown in Figs. 1 and 2.

2.2. Cloning of α_4 subunits of $GABA_A$ receptors

A rat brain oligo dT-primed cDNA library was constructed in the λZAP II-vector (UNIZAP XR Vector Kit, Stratagene, La Jolla, CA, USA) from poly A^+ mRNA isolated from the brains of 8–10-day-old rats as detailed in the protocol from Stratagene. The α_4 subunit of $GABA_A$ receptors was cloned from this cDNA library and its sequence proved to be identical with the respective sequence published previously (Wisden et al., 1991). cDNAs encoding for the α_1, β_3 or γ_{2S} subunits of $GABA_A$ receptors (Fuchs et al., 1995) as well as the cDNA encoding for the α_4 subunit were subcloned into the pCDM8 expression vector (Invitrogen, San Diego, CA, USA) by using standard recombinant DNA procedures (Fuchs et al., 1995). Each plasmid was purified after growth from a single bacterial colony.

2.3. Culturing of human embryonic kidney 293 cells

Human embryonic kidney (HEK) 293 cells (American Type of Culture Collection, Rockville, MD, CRL 1573) were maintained in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin G, and 100 μg/ml streptomycin in 75 cm^2 Petri dishes by using standard cell culture techniques.

HEK 293 cells were transfected with cDNAs encoding for rat α_1, β_3 and γ_{2S} subunits, or α_4, β_3 and γ_{2S} subunits subcloned individually into pCDM8 expression vectors. The ratios for the α, β, and γ subunits used for transfection of 3×10^6 cells with the calcium phosphate precipitation method (Chen and Okayama, 1988) were 12:6:6 μg cDNA. When only α and γ subunits were used for transfection of cells, a cDNA ratio of 2:1 was used. An increase in the proportion of the γ subunit cDNA reduced the growth of the cells as well as specific [^{3}H]Ro 15-4513 binding/mg protein. The medium was changed 20 h after transfection.

2.4. Radioligand binding studies in membranes from HEK cells

Non-transfected HEK 293 cells or cells 96 h after transfection with plasmids encoding for $GABA_A$ receptor

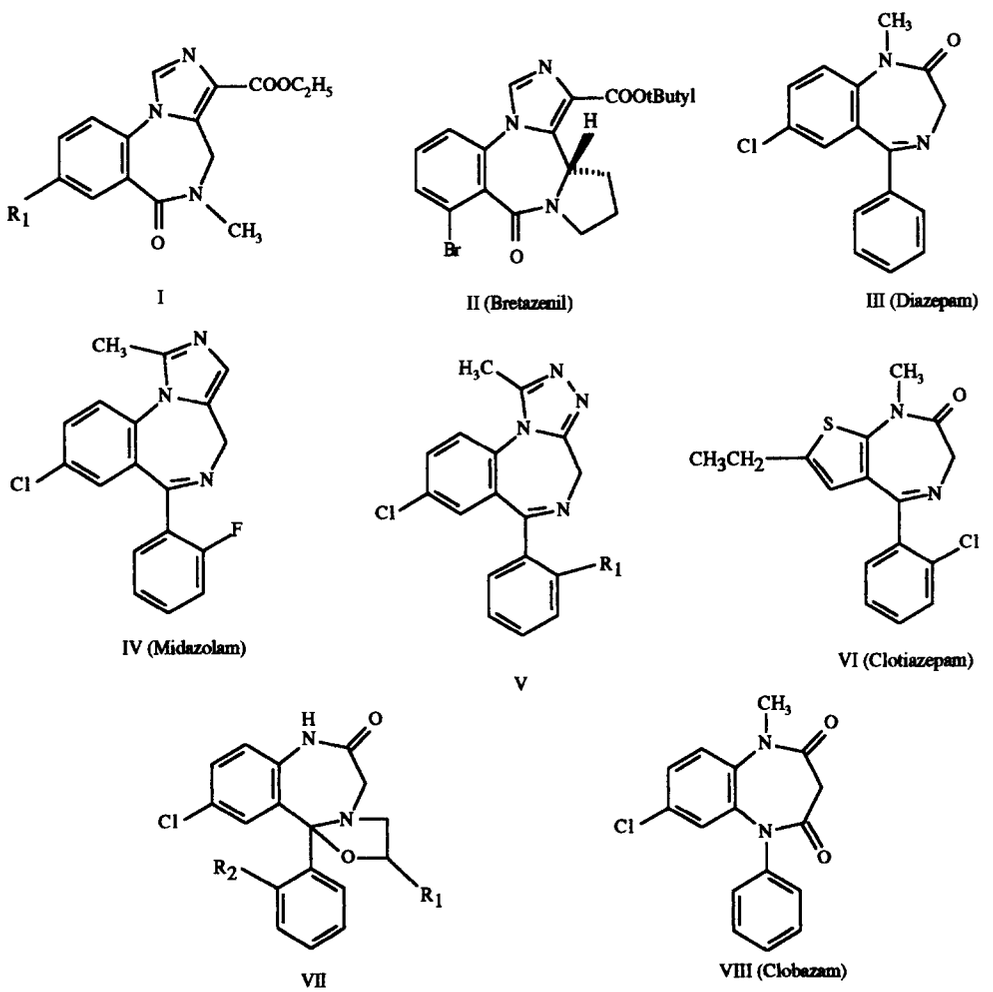


Fig. 1. Structure of the benzodiazepines used in this study. The roman numerals identify structures mentioned in Tables 1 and 2.

subunits were washed twice and then harvested by scraping into phosphate buffered saline. After centrifugation at 12 000 × *g* for 10 min the cell pellets were homogenized in 50 mM Tris-citrate buffer, pH 7.4 by using an Ultraturax, followed by three centrifugation (200 000 × *g* for 20 min) resuspension cycles, and were then used for ligand binding or were stored at −20°C.

For binding studies, both freshly prepared or frozen membranes from non-transfected cells or from cells transfected with plasmids encoding for $GABA_A$ receptor subunits were used with similar results. Frozen membranes were thawed, centrifuged and resuspended in 50 mM Tris-citrate buffer, pH 7.4, at a protein concentration of about 1 mg/ml as measured by the BCA-protein assay kit of Pierce Chem. Co. with bovine serum albumin as standard. Membranes (0.5 ml) were then incubated in a total of 1 ml of a solution containing 50 mM Tris-citrate buffer, pH 7.4, 150 mM NaCl and various concentrations of [^{3}H]Ro 15-4513 in the absence or presence of 100 μM Ro 15-1788 or various concentrations of $GABA_A$ receptor ligands for 90 min at 4°C (Sieghart and Schuster, 1984).

Membranes were then filtered through Whatman GF/B

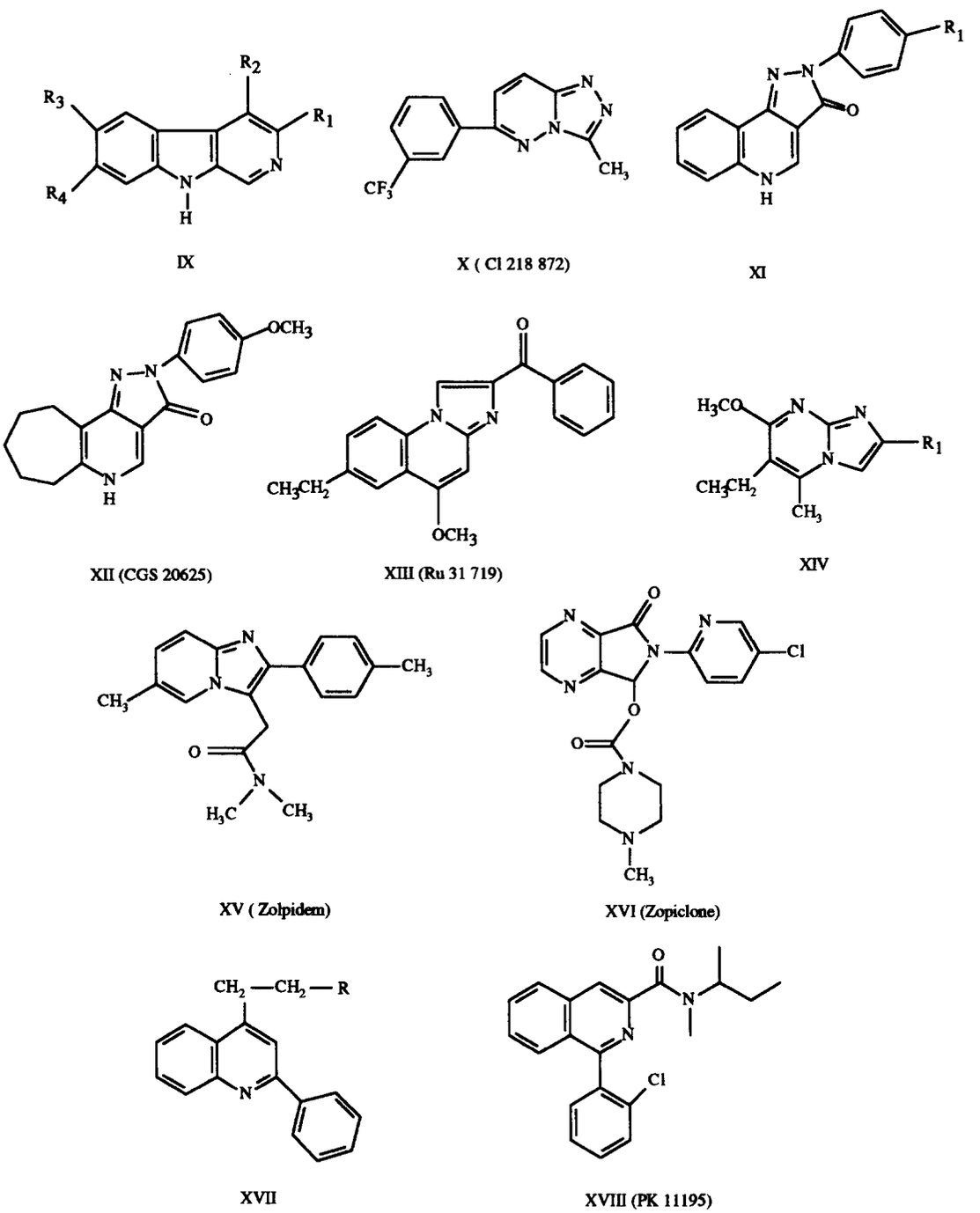


Fig. 2. Structure of other benzodiazepine binding site ligands used in this study. The roman numerals identify structures mentioned in Tables 1 and 2.

filters and the filters were rinsed twice with 5 ml of ice-cold 50 mM Tris-citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after addition of 3.5 ml Hydrofluor (National Diagnostics, NJ, USA) scintillation fluid. Non-specific binding determined in the presence of 100 μM Ro 15-1788 was subtracted from total [^{3}H]Ro 15-4513 binding to obtain specific binding.

3. Results

Membranes from HEK cells transfected with $\alpha_4\beta_3\gamma_{2S}$ or $\alpha_1\beta_3\gamma_{2S}$ subunits were incubated with various concentrations of [^{3}H]Ro 15-4513 in the absence or presence of 100 μM Ro 15-1788, and binding data obtained were subjected to Scatchard analysis. As shown in Table 1 (compound 1), [^{3}H]Ro 15-4513 exhibited a comparably high affinity for $\alpha_4\beta_3\gamma_{2S}$ (K_D of 5.5 ± 1.2 nM, mean ± S.D., $n = 6$) or $\alpha_1\beta_3\gamma_{2S}$ receptors (K_D of 8.1 ± 0.6 nM, mean ± S.D., $n = 5$). The B_{max} values, however, were significantly different in $\alpha_4\beta_3\gamma_{2S}$ (B_{max} of 128 ± 10 fmol/mg protein, mean ± S.D., $n = 6$) and $\alpha_1\beta_3\gamma_{2S}$ (B_{max} of 1105 ± 237 fmol/mg protein, mean ± S.D., $n = 5$) transfected cells, indicating a comparably weak expression of recombinant $\alpha_4\beta_3\gamma_{2S}$ receptors under the conditions used. The extent of expression of these receptors could not be improved by changing the $GABA_A$ receptor subunit cDNA ratio used for transfection of the cells.

In other experiments, the potency of a total of 30 benzodiazepine binding site ligands for the inhibition of [^{3}H]Ro 15-4513 binding to membranes from $\alpha_4\beta_3\gamma_{2S}$ or $\alpha_1\beta_3\gamma_{2S}$ transfected HEK cells was compared. For compounds exhibiting a high affinity for $\alpha_4\beta_3\gamma_{2S}$ receptors, IC_{50} values were determined and transformed into K_i values using the Cheng-Prusoff relationship (Cheng and Prusoff, 1973). For compounds exhibiting an extremely low affinity for these receptors, single point measurements using 10 μM concentrations of the benzodiazepine binding site ligands were performed.

From all benzodiazepines investigated, only Ro 15-4513 and its structural analogues, the imidazobenzodiazepines Ro 15-1788 (Table 1, compound 2) and bretazenil (Table 1, compound 3) exhibited a high affinity for $\alpha_4\beta_3\gamma_{2S}$ receptors. The affinity of bretazenil and Ro 15-1788 for these receptors, however, was lower than that of Ro 15-4513, and was 100-fold and 50-fold lower than that for $\alpha_1\beta_3\gamma_{2S}$ receptors, respectively. In contrast, all the 1,4-benzodiazepines investigated (Table 2, compounds 16–21), the 1,4-thienodiazepine clotiazepam (Table 2, compound 22), and the 1,5-benzodiazepine clobazam (Table 2, compound 23) did not or only marginally inhibit [^{3}H]Ro 15-4513 binding to $\alpha_4\beta_3\gamma_{2S}$ receptors under conditions where these compounds were able to strongly inhibit binding of this compound to $\alpha_1\beta_3\gamma_{2S}$ receptors.

From all β-carbolines (Fig. 2, structure IX) investigated, only DMCM (Table 1, compound 4) exhibited a high affinity for $\alpha_4\beta_3\gamma_{2S}$ receptors and this affinity was similar to that for $\alpha_1\beta_3\gamma_{2S}$ receptors. In contrast, the structural analogue β-CCP (Table 1, compound 7) exhibited a 100-fold, β-CCM (Table 1, compound 5) or β-CCE (Table 1, compound 6) a 1000-fold and abecarnil (Table 1, compound 9) a 5000-fold lower affinity for $\alpha_4\beta_3\gamma_{2S}$ than for $\alpha_1\beta_3\gamma_{2S}$ receptors. And finally, the β-carboline FG 7142 (Table 1, compound 8), only marginally inhibited [^{3}H]Ro 15-4513 binding to receptors consisting of $\alpha_4\beta_3\gamma_{2S}$ subunits at 10 μM concentrations.

All pyrazoloquinolines (Table 1, compounds 10–12) investigated and the pyrazolopyridine CGS 20625 (Table 1, compound 13) exhibited a relatively high affinity for $\alpha_4\beta_3\gamma_{2S}$ receptors. Nevertheless, the affinity of these compounds was 40–200-fold lower than that for $\alpha_1\beta_3\gamma_{2S}$ receptors.

Other compounds, however, were quite inactive on $\alpha_4\beta_3\gamma_{2S}$ receptors under conditions where these compounds significantly inhibited [^{3}H]Ro 15-4513 binding to receptors consisting of $\alpha_1\beta_3\gamma_{2S}$ subunits. Thus, the triazolopyridazine Cl 218872 (Table 1, compound 14), the imidazopyridine zolpidem (Table 1, compound 15), the cyclopyrrolone zopiclone (Table 2, compound 24), the quinolines PK 8165 and PK 9084 (Table 2, compounds 29 and 30) and the imidazopyrimidine Ru 33 203 (Table 2, compound 27) only marginally inhibited [^{3}H]Ro 15-4513 binding to $\alpha_4\beta_3\gamma_{2S}$ receptors. Other imidazopyrimidines (Table 2, compounds 26 and 28), however, or the imidazoquinoline Ru 31 719 (Table 2, compound 25), although still rather ineffective, seemed to exhibit a slightly higher potency for inhibition of [^{3}H]Ro 15-4513 binding to $\alpha_4\beta_3\gamma_{2S}$ receptors than the above mentioned compounds. The peripheral benzodiazepine binding site ligand PK 11195 (Table 2, compound 31) did not inhibit [^{3}H]Ro 15-4513 binding to $\alpha_4\beta_3\gamma_{2S}$ receptors and, in agreement with previous experiments with brain membranes (Hirsch et al., 1988), only weakly inhibited binding to $\alpha_1\beta_3\gamma_{2S}$ receptors.

In other experiments, the potency of several benzodiazepine binding site ligands for displacement of [^{3}H]Ro 15-4513 binding from membranes of $\alpha_4\gamma_{2S}$ transfected HEK cells was investigated. As shown in table 1, the affinity of the benzodiazepines Ro 15-4513 and bretazenil was comparable for receptors consisting of $\alpha_4\gamma_{2S}$ or $\alpha_4\beta_3\gamma_{2S}$ subunits. The affinity of Ro 15-1788, DMCM or CGS 20625 for $\alpha_4\gamma_{2S}$ receptors, however, was lower than that for $\alpha_4\beta_3\gamma_{2S}$ receptors. As with receptors consisting of $\alpha_4\beta_3\gamma_{2S}$ subunits, diazepam was not able to inhibit [^{3}H]Ro 15-4513 binding to membranes from $\alpha_4\gamma_{2S}$ transfected HEK cells (experiments not shown). Because the total amount of radioactivity specifically bound to membranes of $\alpha_4\gamma_{2S}$ transfected cells was low (B_{max} value ($n = 6$) of 68 ± 13 fmol/mg protein), and specific binding was variable (35 ± 13% of total binding at 5 nM concentration of the radioligand, mean ± S.D., $n = 6$), the po-

tency of other compounds for the displacement of [^{3}H]Ro 15-4513 from membranes of $\alpha_4\gamma_{2S}$ transfected cells was not investigated.

4. Discussion

In the present study, the potency of 30 benzodiazepine binding site ligands for the inhibition of [^{3}H]Ro 15-4513 binding to recombinant $GABA_A$ receptors consisting of α_4, β_3, and γ_{2S} subunits was investigated and compared with that for receptors consisting of α_1, β_3, and γ_{2S} subunits. Most of the compounds investigated exhibited a high potency for inhibition of [^{3}H]Ro 15-4513 binding to membranes from $\alpha_1\beta_3\gamma_{2S}$ transfected HEK cells and the potency observed was comparable with that obtained with brain membranes (Haefely et al., 1985; Gardner et al., 1993) or with α_1 subunit containing recombinant receptors (for review see Sieghart, 1995).

The potency of these compounds for inhibition of [^{3}H]Ro 15-4513 binding to $\alpha_4\beta_3\gamma_{2S}$ receptors was distinct, however. In agreement with previous results (Wisden et al., 1991; Wieland and Lüddens, 1994), Ro 15-4513 exhibited a high and Ro 15-1788 a relatively low affinity for receptors on $\alpha_4\beta_3\gamma_{2S}$ transfected cells, whereas the classical 1,4-benzodiazepine diazepam as well as the triazolopyridazine CI 218872 and the imidazopyridine zolpidem exhibited no affinity at all for these receptors. In the present study these results were extended by demonstrating that the benzodiazepine bretazenil, the β-carboline DMCM, the pyrazolopyridine CGS 20625, and all the pyrazoloquinolines investigated exhibited a relatively high affinity for $\alpha_4\beta_3\gamma_{2S}$ receptors, although the affinity of these compounds for this receptor was lower than that for $\alpha_1\beta_3\gamma_{2S}$ receptors. In contrast to the β-carboline DMCM, the β-carbolines β-CCM, β-CCE, β-CCP and abecarnil exhibited a significantly reduced affinity, whereas FG 7142 exhibited no affinity at all for $\alpha_4\beta_3\gamma_{2S}$ receptors. All other compounds investigated, however, such as several 1,4-benzodiazepines, the 1,4-thienodiazepine clotiazepam, the 1,5-benzodiazepine clobazam, the cyclopyrrolone zopiclone, the imidazoquinoline Ru 31719, and several quinolines and imidazo-pyrimidines, did not or only weakly inhibit binding of [^{3}H]Ro 15-4513 to $\alpha_4\beta_3\gamma_{2S}$ receptors. These data confirm previous conclusions that $\alpha_4\beta_3\gamma_{2S}$ receptors exhibit unique benzodiazepine binding properties, resembling those of receptors consisting of $\alpha_6\beta_2\gamma_2$ subunits (Wisden et al., 1991) or of 'diazepam-insensitive $GABA_A$ receptors' found in cerebellum (Sieghart et al., 1987; Wong et al., 1992b, 1993).

Recently, it has been demonstrated that a point mutation in the α_4 subunit, changing an arginine at the position 99 to histidine, resulted in recombinant receptors exhibiting a high affinity for diazepam, Ro 15-1788, zolpidem and β-CCM, and in a significantly enhanced but still relatively low affinity for CI 218872 (Wieland and Lüddens, 1994). In contrast, a similar mutation in the corresponding position 100 in the α_6 subunit resulted in recombinant receptors which, although exhibiting an enhanced affinity for diazepam, Ro 15-1788, and β-CCM, exhibited a still extremely low affinity for CI 218872 and no affinity at all for zolpidem (Wieland and Lüddens, 1994). These data indicate that an exchange of a single amino acid at a homologous position is not sufficient to change the benzodiazepine binding properties of recombinant α_4 or α_6 subunit containing receptors in parallel, and suggest that the pharmacology of α_4 and α_6 subunit containing receptors might be different. The present identification of additional compounds exhibiting high or low affinity for the benzodiazepine binding site of $\alpha_4\beta_3\gamma_2$ receptors could help to further distinguish between α_4 and α_6 subunit containing receptors and to investigate the benzodiazepine binding pocket of $GABA_A$ receptors by mutagenesis studies (Wieland and Lüddens, 1994).

Previous results have indicated that receptors consisting of $\alpha_1\gamma_2$ subunits exhibited benzodiazepine binding properties similar to that of $\alpha_1\beta_x\gamma_2$ receptors (Wong et al., 1992a). In the present study it was demonstrated that all compounds investigated exhibited an affinity that was comparable for $\alpha_4\gamma_{2S}$ or $\alpha_4\beta_3\gamma_{2S}$ receptors, but did not resemble the affinity for $\alpha_1\beta_3\gamma_{2S}$ receptors. These data support previous conclusions, that the benzodiazepine binding site can be formed by α and γ subunits in the absence of β subunits (Wong et al., 1992a), and that the pharmacology of this binding site predominantly is determined by the α subunit of $GABA_A$ receptors (Sieghart, 1995).

In conclusion, the present data indicate that compounds from at least four different structural classes, the imidazobenzodiazepines, the β-carbolines, the pyrazoloquinolines, and the pyrazolopyridines, exhibit a high affinity for the benzodiazepine binding site of $GABA_A$ receptors consisting of $\alpha_4\beta_3\gamma_{2S}$ subunits. Electrophysiological experiments will have to be performed to investigate whether these compounds act as agonists, antagonists or inverse agonists on $\alpha_4\beta_3\gamma_{2S}$ receptors. Variation of the structure of these compounds, however, could lead to the development of compounds selectively interacting with $\alpha_4\beta_3\gamma_{2S}$ subunit containing receptors.

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Note added in proof

After submission of this manuscript an additional study became available (Yang et al., 1995), reporting the benzodiazepine binding site affinities of 13 of the compounds investigated in the present study but using human $\alpha_4\beta_2\gamma_{2L}$

instead of rat $\alpha_4\beta_3\gamma_{2S}$ receptors. Results obtained in this study were comparable with those of the present study, reflecting the high homology of rat and human $GABA_A$ receptor subunits and the comparably low effect of the type of the β or γ subunit on the benzodiazepine binding site properties of recombinant $GABA_A$ receptors (Sieghart, 1995).

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