Eur J Med Chem (1996) 31, 575–587

Synthesis and benzodiazepine receptor binding of some imidazo- and pyrimido[2,1-*b*]benzothiazoles

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(Received 18 August 1995; accepted 31 January 1996)

Summary — A series of substituted imidazo[2,1-*b*]benzothiazoles **2a–u** was synthesized and the compounds evaluated for their affinity at the central benzodiazepine receptors. Substitution at the 7-position generally resulted in a decreased ligand affinity whereas a significant increase was observed for 5-substituted compounds. The intrinsic efficacy of selected high-affinity ligands **2j,k,q**, as well as some previously reported pyrimido[2,1-*b*]benzothiazoles **1**, was measured in vitro through the determination of the GABA ratio and [^{35}S]TBPS displacement. Consistent with a partial inverse agonist profile, the benzothiazole derivatives **2j,k,q** increased [^{35}S]TBPS binding. For compounds **1c** and **1d**, a discrepancy between GABA ratio and [^{35}S]TBPS binding data was observed. Only the latter assay was in full agreement with the pharmacological data, which indicated an inverse agonist and a partial agonist profile for **2k,q** and **1c,d** respectively. The affinity and intrinsic activity data of compounds **1c,d** and **2j,k,q** are discussed in the light of the recently proposed pharmacophore model by Skolnick/Cook; in particular, the agonistic activity of **1c,d** is interpreted on the basis of a possible interaction of substitutents in position 6 with the receptors lipophilic area L3 of Skolnick/Cook, whereas the observed inverse agonist profile of **2j,k,q** is explained taking into account their structural analogy with the well known proconvulsant β-CCE.

imidazo[2,1-*b*]benzothiazole / pyrimido[2,1-*b*]benzothiazole / [^{35}S]TBPS binding / GABA ratio / molecular modeling / convulsant activity

Introduction

Since the introduction of the benzodiazepines (BZs) into clinical practice, many advances have been made in understanding the molecular mechanism(s) by which this structural class of drugs produces its effects. Specific, high-affinity binding sites for BZs in the mammalian CNS have been identified on a receptor protein (BZR) which is part of the GABA/Cl-ionophore complex [1]. It should be taken into account that recent molecular biological studies have demonstrated that the BZR pharmacology is complicated by the existence of multiple BZR subtypes; unfortunately, their distribution and physiological roles still have to be fully determined, and moreover only few ligands acting specifically at one receptor subtype are known [2]. Moreover, an impressive number of structurally novel agents, which do not contain the 1,4-benzodiazepine nucleus, have been shown to interact with the BZRs. The pharmacological properties of these ligands range from anxiolytic/anticonvulsant for agonists, anxiogenic/convulsant for inverse agonists, to nil efficacy for antagonists. However, in vitro and in vivo assays have shown that most of the available ligands present some limitations in terms of receptor selectivity, unwanted side-effects and pharmacokinetic profile. Thus, the search continues for new BZRs ligands with enhanced selectivity, safety, and efficacy [1, 3, 4]. A rational design of new BZRs ligands should be based on reliable pharmacophore models for recognition and activation of the BZRs. Thus, several different pharmacophore models have been proposed on the basis of binding data at multiple BZR subtypes displayed by various structural families of BZRs ligands; such models could be useful starting points for identification of the molecular determinants of recognition and activation at single BZR subtypes when sufficient biological data become available for single receptor subtypes. Despite the differences in the methodological approaches used, some patterns common to all the known models are emerging. One feature common to most models is the existence of two proton accepting groups separated by an average distance of ~3.6 Å and one or more lipophilic

region(s), which presumably interact with hydrophobic pocket(s) in the BZRs. Moreover a further requirement for optimal binding to BZRs is the ability of the ligand to assume a planar or pseudoplanar topography [5–19].

In this context, in a previous paper [20] we have reported the synthesis of the 3-alkoxycarbonyl-4*H*-pyrimido[2,1-*b*]benzothiazol-4-one series **1**, among which **1b ,1c,** and **1d** (see chart 1) bound to BZRs with potency slightly greater than chlordiazepoxide (IC_{50} values of 260, 200 and 170 nM respectively). Molecular orbital calculations on **1** and related compounds have been carried out and their overall low binding affinity has been explained, at least partially, in terms of unfavorable distances and orientation of the three putative BZRs anchoring groups, namely two hydrogen bond (HB)-accepting groups and the center of an aromatic ring. Such unsatisfactory results prompted us to design a new series of BZRs ligands carrying the two HB accepting groups at a more appropriate distance. We reasoned that modifications of structure **1** leading to structures with a shorter distance between the N(1) and the oxygen of the ester carbonyl (both HB-accepting groups), and the removal of the lactam carbonyl to avoid alternative nonproductive binding, might result in improved binding affinities. These arguments induced us to consider the imidazo[2,1-*b*]benzothiazole ring system **2** as a lead structure for studying structure–affinity relationships of BZR (see chart 1).

A literature survey reveals that the benzothiazole series **2** have been tested for BZR affinity and show IC_{50} values ranging from 50–7000 nM [21]. Although some ester derivatives of this series exhibited high in vitro receptor affinity, they proved inactive when tested in vivo as anxiolytics. This result has been ascribed to unfavorable pharmacokinetic features [21]. The effects of fused benzene ring substituents in the benzothiazole series **2** on binding and pharmacological properties have not yet been thoroughly explored. Therefore, we attempted to gain further insight into the SAR of the benzothiazole series **2** by synthesizing and testing a number of benzothiazole compounds **2** substituted at positions 5–8. In addition, we have tried to predict the in vitro efficacy of the most active compounds **1** and **2** by determining, for comparative purpose, both the 'GABA ratio' [ie (IC_{50} without GABA/IC_{50} with GABA)] and [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding. Both assays, and the GABA ratio in particular, have been employed in the past to predict the efficacy of BZRs ligands, but, to the best of our knowledge, a direct comparison of the predictive capability of these in vitro procedures has never been carried out. Thus, we have found some compounds with high affinity for BZRs whose selectivity has not yet been defined.

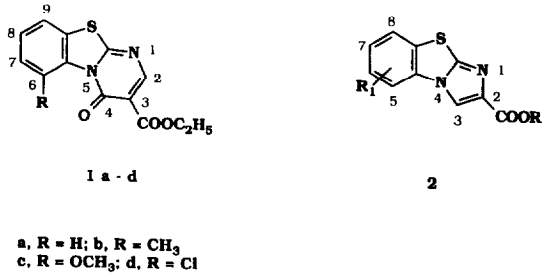
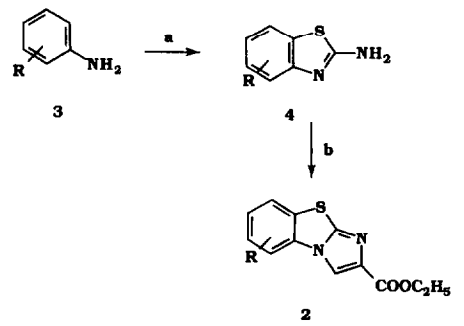


Chart 1. Structural formulas of compounds **1a–d** and **2**.

Chemistry

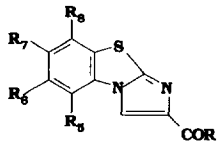
The general synthetic procedure employed to prepare compounds **2e–p** (table I) is shown in scheme 1. It follows essentially the method previously reported [21] for the synthesis of **2b,d** and begins with the preparation of the appropriate 2-aminobenzothiazoles **4**. Compounds **4** are then reacted with ethyl bromopyruvate, affording an intermediate hydrobromide which was subsequently cyclized to **2** in refluxing EtOH or DMF (for **2i** and **2n**). According to literature methods [21], benzothiazoles **2q** and **2r** were obtained by treatment of the corresponding acids with boron trifluoride etherate in methanol or oxalyl chloride/benzyl alcohol respectively. Structural assignments of compounds **2** were based on IR, ^{1}H NMR and mass spectral data. In particular, the ^{1}H NMR spectra of the new derivatives **2e,h,i,o,p** as well as the previously reported compounds **2a–d,f,g,m** were characterized by a sharp singlet at δ 8.3–8.9 which was due to the proton attached to the C(3) carbon.

As for the 5-substituted compounds **2j–l,n,q,r**, structural assignment was obtained by comparing

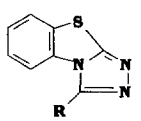


Scheme 1. (a) KSCN/Br_2, CH_3COOH; (b) $BrCH_2COCOO$-C_2H_5, EtOH.

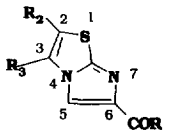
Table I. Physical data of 2-alkoxycarbonyl-imidazo[2,1-*b*]benzothiazoles **2a–u**, triazoles **7a,b** and imidazo[2,1-*b*]thiazoles **8a–c**.



2a-u



7a,b



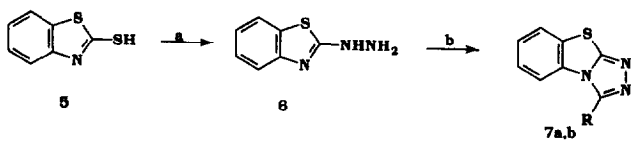
8a-c

Compd	*R*	R_2	R_3	R_5	R_6	R_7	R_8	*Yield (%)*	*Mp (°C)*	*Formula or ref*
2a	OCH_3			H	H	H	H			a
2b	OC_2H_5			H	H	H	H			a
2c	$OCH_2C_6H_5$			H	H	H	H			a
2d	OC_2H_5			H	H	C_2H_5	H			a
2e	OC_2H_5			H	H	F	H	15	158–160[a]	$C_{12}H_9FN_2O_2S$
2f	OC_2H_5			H	H	Cl	H			b
2g	OC_2H_5			H	H	Br	H			b
2h	OC_2H_5			H	H	OCH_3	H	23	130–131[d]	$C_{13}H_{12}N_2O_3S$
2i	OC_2H_5			H	H	NO_2	H	7	269–272[e]	$C_{12}H_9N_3O_4S$
2j	OC_2H_5			Cl	H	H	H			c
2k	OC_2H_5			OCH_3	H	H	H	30	138–140[f]	$C_{13}H_{12}N_2O_3S$
2l	OC_2H_5			CH_3	H	H	H	26	159–160[d]	$C_{13}H_{12}N_2O_2S$
2m	OC_2H_5			H	CH_3	CH_3	H			b
2n	OC_2H_5			Cl	H	Cl	H	14	260–262[g]	$C_{12}H_8Cl_2N_2O_2S$
2o	OC_2H_5			H	Cl	Cl	H	16	235–237[h]	$C_{12}H_8Cl_2N_2O_2S$
2p	OC_2H_5			H	H	Cl	Cl	65	230–232[h]	$C_{12}H_8Cl_2N_2O_2S$
2q	OCH_3			Cl	H	H	H	50	202–205	$C_{11}H_7ClN_2O_2S$
2r	$OCH_2C_6H_5$			Cl	H	H	H	22	128-130[g]	$C_{17}H_{11}ClN_2O_2S$
2s	NH_2			H	H	H	H			c
2t	$NHNH_2$			H	H	H	H	33	>267	$C_{10}H_8N_4OS$
2u	$NHNH_2$			Cl	H	H	H	20	272[h]	$C_{10}H_7ClN_4OS$
7a	H							29	172–174	$C_8H_5N_3S$
7b	C_6H_5							37	151–153[g]	$C_{14}H_9N_3S$
8a	OC_2H_5	H	H							a
8b	OC_2H_5	H	C_6H_5					26	163–164[i]	$C_{14}H_{12}N_2O_2S$
8c	OC_2H_5	H	4-Cl-C_6H_4					14	187–188[i]	$C_{10}H_{11}ClN_2O_2S$

[a]Reference 21; [b]reference 22; [c]reference 23; [d]from EtOAc/$CHCl_3$; [e]from toluene; [f]from EtOAc; [g]from petroleum ether/EtOAc; [h]from $CHCl_3$/EtOH; [i]from Ligroin/ EtOAc.

^{13}C NMR chemical shifts of quaternary and tertiary carbons to the corresponding ones observed for 5-unsubstituted compounds **2**. Furthermore, the 2D NOESY ^1H-NMR spectrum of **2l** was particularly diagnostic for the presence of crosspeaks between the protons of the methyl group linked at C(5) and the proton at C(3), thus unambiguously confirming the structural assignment.

To assess the importance of the ester function and the fused benzene ring for receptors binding, we prepared and screened amide **2s**, hydrazides **2t,u**, triazolobenzothiazoles **7a,b** as well as imidazothiazoles **8a–c**. Compounds **2s** and **2t,u** were obtained starting with **2b** and **2j**, by ammonolysis and hydrazinolysis respectively. The synthetic route to triazolobenzothiazoles **7a,b**, outlined in scheme 2, involved the preparation of 2-hydrazinobenzothiazole **6** followed by treatment with trimethyl orthoformate or trimethyl orthobenzoate. Physical and microanalytical data of newly prepared compounds are listed in table I.



Scheme 2. (a) NH_2NH_2; (b) $HC(OMe)_3$ for **7a** and $C_6H_5C(OMe)_3$ for **7b**.

Biological results and discussion

The ability of the benzothiazole series of compounds **2** to interact with the BZRs was investigated by a binding assay using [^{3}H]flunitrazepam as radioligand and rat membranes from brain tissues as receptor source. The percentage of inhibition of specific [^{3}H]flunitrazepam binding was determined by using a 40 μM concentration of the tested compounds followed by the determination of IC_{50} only for the most active ones (percentages of inhibition greater than 75%). Two classical benzodiazepines in clinical use, namely chlordiazepoxide and diazepam, were included as reference compounds. The measured binding affinities are shown in tables II and III. For the most active compounds **2j,k,q**, the inhibition of [^{3}H]flunitrazepam binding to mouse membranes was also determined.

Structure–BZRs affinity relationships

Using compound **2b** as a reference, we first tried to evaluate the influence of electronic (σ) and lipophilic (π) effects on the biological activity by suitable substitutions on positions -5, -6, -7, and -8 of the aromatic ring.

As for the 7-substituted imidazobenzothiazoles 7-fluoro (**2e**), 7-chloro (**2f**) and 7-bromo (**2g**), representing electron-withdrawing (+σ) and hydrophobic (+π) substituents [24], we observed an increase in affinity only with **2e** which carries a small and electron-withdrawing atom, fluorine. The 7-methoxy (**2h**), 7-ethyl (**2d**) and 7-nitro (**2i**) congeners, presenting −σ, −π, −σ, +π, and +σ, −π substituents respectively [24], showed very low binding affinities. The effect of the aromatic substitution at the 5-position of the imidazo-

Table II. Effect of in vitro addition of some 2-(alkoxycarbonyl)-4*H*-imidazo[2,1-*b*]benzothiazoles **2a–g,i–m,o,q,r**), and imidazo[2,1-*b*]thiazoles **8b** on [^{3}H]flunitrazepam binding and 5 μM muscimol-stimulated $^{36}Cl^-$ uptake in rat and mouse cortical membranes.

Compound	*[^{3}H]Flunitrazepam binding*		*$^{36}Cl^-$ uptake*[c]			
	IC_{50}[a] *(nM)*	*GABA ratio*[b]	*1 μM*	*10 μM*	*50 μM*	*100 μM*
2a	60[d]					
2b	120[d]					
2c	50[d]					
2d	4000[d]					
2e	51 ± 4	0.88	82 ± 5	80 ± 7		74 ± 8
2f	148 ± 8					
2g	310 ± 7.5					
2i	2900 ± 80					
2j	36 ± 2	1.00	74 ± 5	70 ± 4		68 ± 4
	116 ± 7[e]					
2k	40 ± 2	1.00	97 ± 6	103 ± 11	103 ± 4	85 ± 5
	85 ± 4[e]					
2l	97 ± 5	0.81				
2m	340 ± 26					
2o	104 ± 7	1.13				
2q	62 ± 2	1.10	96 ± 4	92 ± 3	87 ± 5	80 ± 6
	84 ± 10[e]					
2r	137 ± 6					
8b	1200					
Chlordiazepoxide	500 ± 20					
Diazepam	5 ± 0.4					
DMCM	0.8 ± 0.1		76 ± 2	75 ± 8	72 ± 3	71 ± 4

[a]Concentration necessary for 50% inhibition (IC_{50}); data are means ± SD of two determinations. [b]GABA ratio = IC_{50} without GABA/IC_{50} with GABA. [c]Data are the means ± SEM of four separate experiments. [d]Reference 21. [e]Data, from mouse cortical membranes, are the means ± SEM of three separate experiments.

benzothiazole nucleus was investigated by synthesizing 5-chloro (**2j**), 5-methyl (**2l**), and 5-methoxy (**2k**) congeners. The introduction of a chloro or methoxy substituent led to an enhancement of the radioligand displacement. The same effect, although to a lesser degree, was observed with **2l**.

The low affinity showed by all the disubstituted compounds might indicate that there are steric constraints to multiple aromatic substitution which cannot be overlooked. The above findings suggested that, in the more thoroughly investigated ethyl ester series, substitution at the 5-position led to compounds with the highest BZRs binding affinity (<100 nM). It is interesting to note that in the pyrimidobenzothiazole series **1**, substitution at the 6-position (the topological equivalent to position 5 in **2**) with chlorine, methoxy and methyl groups also afforded the most active compounds [20, 25].

To define the importance of the ester functionality for receptor binding we prepared and evaluated the 2-methoxycarbonyl- and 2-benzyloxycarbonyl-5-chloroimidazobenzothiazoles **2q,r**. In neither case was potency improvement over **2j** observed. Furthermore, in order to evaluate the effect of other HB acceptor groups, we considered more drastic modifications by changing the ester functionality to an amide (**2s**) or hydrazide (**2t,u**), the imidazole moiety to triazole (**7a,b**) and, finally, to test the effect of the benzene ring we also prepared the thiazole compounds (**8a–c**). In all cases, very poor affinity was observed confirming similar findings for other series of BZRs ligands. The loss of BZRs affinity observed for compounds **8a–c** could be taken as evidence of the important role played by the benzene ring which should likewise be involved in a hydrophobic (or π–π) interaction with the receptor.

On the basis of our findings, it can be concluded that a significant improvement in affinity over the known compound **2b** can be obtained by introducing a chloro (**2j**) or methoxy (**2k**) group at the 5-position. Furthermore, it is interesting to note that **2j** and **2k** showed binding affinities higher than that reported for the most active known congeneric compound **2c**.

An estimate of the efficacy of the high affinity compounds **1** and **2** was made by determining the GABA ratio. The most active imidazobenzothiazoles **2** (table II) showed a GABA ratio close to unity whereas the pyrimidobenzothiazoles **1b,d** and **1c** displayed GABA ratios respectively greater and lower than 1 [20, 25]. This suggests that compounds **2** and **1c** are either antagonists or inverse agonists, whereas compounds **1b,d** could be agonists. However, the usefulness of the GABA ratio as a predictor of antagonistic or inverse agonistic activity has been often questioned [8, 26]; in fact, only agonists have systematically displayed GABA ratios greater than unity.

Table III. Inhibition of [^{3}H]flunitrazepam binding by 40 μM of some 2-(alkoxycarbonyl)-4*H*-imidazo[2,1-*b*]-benzothiazoles **2h,n,p,s–u**), triazoles **7a,b** and imidazo[2,1-*b*]thiazoles **8a–c** in rat cortical membranes.

Compound	*[^{3}H]Flunitrazepam binding (% of inhibition)*
2h	64 ± 6
2n	71 ± 2
2p	34 ± 0.5
2s	42 ± 0.8
2t	64 ± 2
2u	56 ± 2
7a	NA
7b	80 ± 4
8a	19 ± 0.2
8c	45 ± 2

NA: Ineffective up to 10 000 nM. Data are the means of four separate experiments.

Therefore, to characterize more precisely the pharmacological profile of the most active compounds at the $GABA_A$ receptor complex, we studied their effect on [^{35}S]TBPS binding in mouse cerebral cortex membranes .

[^{35}S]TBPS is a specific ligand for a site located at the level of the chloride channel coupled to the $GABA_A$ receptor [27], and changes in its binding are correlated with parallel changes in the function of the GABA-coupled chloride channel; in vivo administration of drugs which alter the cognitive, emotive and motor functions, such as benzodiazepine inverse agonists, enhanced the total number of [^{35}S]TBPS binding sites in a dose-dependent manner, an opposite effect to that produced by benzodiazepines and GABA agonists [28]. Figure 1 shows that some of the tested compounds, namely **2j,k,q**, increased [^{35}S]TBPS binding in a concentration-dependent manner, an effect similar to that induced by the β-carboline derivative DMCM (3-carbomethoxy-4-ethyl-6,7-dimethoxy-9*H*-pyrido[4,3-*b*]indole), a negative modulator of the $GABA_A$ receptor function. Consistent with these findings, analogs **2j,k,q** decreased muscimol-stimulated $^{36}Cl^-$ uptake by 20–30% at 100 μM (table II).

With compounds **1c,d** a slight decrease of [^{35}S]-TBPS binding was observed which is not consistent with the measured GABA ratio of less than unity for **1c**. Pharmacological profile (vide infra) are in full agreement with the [^{35}S]TBPS binding data.

Pharmacology.

The finding that **2j,k,q,** like the convulsant DMCM, increased [^{35}S]TBPS binding in vitro, suggested that these compounds may generate pharmacological

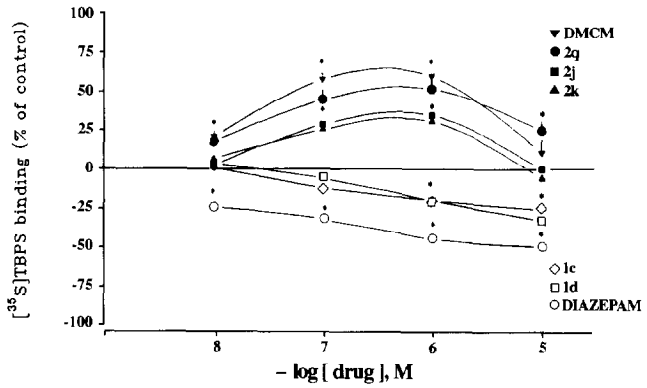


Fig 1. Differential changes of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding by **2j**,**k**,**q**, **1c** and **1d**. [^{35}S]TBPS binding was measured in fresh, unwashed membrane preparations from mouse cerebral cortex with 2 nM [^{35}S]TBPS. Data are expressed as percentage decrease in binding from control values and are the means ± SEM from four separate experiments. *$p < 0.05$ vs control.

effects similar to those found for negative modulators of GABAergic transmission. As expected, the acute ip administration of increasing doses of **2q** (10–20 mg/kg) induced tonic/clonic seizures within a few minutes in mice (table IV). This effect is antagonized by the specific benzodiazepine receptor antagonist flumazenil. In fact, the ip injection of 5 mg/kg of flumazenil, 15 min before **2q** (15 mg/kg ip), completely abolished the convulsant effect elicited by this compound.

In contrast, **2j** and **2k** (20 mg/kg ip) failed to induce convulsions in mice (table IV). However, **2j** and **2k** (20–30 mg/kg ip), when administered 45 min after a subconvulsant dose (150 mg/kg sc) of isoniazid [29], increased the mortality and percentage of animals that had convulsions. Moreover, they decreased the latency (–15 min; –20 min) of isoniazid-induced seizures (table IV).

Based on the latter results, **2k** and **2j** may be classified as partial inverse agonists like the β-carboline derivatives FG 7142 and β-CCE [28, 30, 31] which

Table IV. Proconvulsant and convulsant activity of compounds **2j**[a], **2k**[b], and **2q**[c] in control and isoniazid-treated mice.

Compound	*Dose (ip) (mg/kg)*	*Convulsions*			*Mortality*		
		Onset (min)	*No of animals*	*%*	*Onset (min)*	*No of animals*	*%*
2q	5	–	–	–	–	–	–
	10	1.30 ± 0.1	12/28	43	–	0/28	0
	15	1.54 ± 0.3	21/28	75	2.20 ± 0.3	7/28	25
	20	1.00 ± 0.2	25/25	100	1.50 ± 0.1	15/25	60
	15 + 5 flumazenil	–	0/16	0	–	0/16	0
2k	20	–	–	–	–	–	–
Isoniazid	150	79 ± 6	5/25	20	81 ± 2	3/25	12
2k	20 + 150 Isoniazid	59 ± 1[d]	20/30[e]	66	71 ± 9	11/30	36
2j	20	–	–	–	–	–	–
Isoniazid	150	62 ± 4	5/21	24	–	0/21	0
2j	20 + 150 Isoniazid	73 ± 7.5	9/25	36	–	0/25	0
2j	30 + 150 Isoniazid	47 ± 0.3[d]	13/28	45	–	0/28	0

[a]**2j** (20–30 mg/kg ip) was administered 45 min after isoniazid (200 mg/kg sc). Animals were observed for 180 min, during which the latency of tonic clonic seizures and death was recorded. Data are the mean ± SEM of three separate experiments. [b]Animals were injected ip with **2k** (20 mg/kg) 45 min after isoniazid (200 mg/kg sc) and they were observed for 180 min, during which the latency of tonic clonic seizures and death was recorded. Data are the mean ± SEM of three separate experiments. [c]The mice were injected with **2q** (5–20 mg/kg ip) and they were observed for 60 min, during which the latency of tonic clonic seizures and death was recorded. Flumazenil was given 15 min before **2q** to mice. Each value represents the mean ± SEM of three separate experiments. [d]$P < 0.01$ vs isoniazide-treated mice (Student's *t* test). [e]$P < 0.025$ vs isoniazid-treated mice (Fisher's exact probability test).

Table V. Differential efficacy of compounds **1c,d**[a,b] on the convulsions and death elicited by isoniazid and PTZ in mice.

Compound	*Dose (ip) (mg/kg)*	*Convulsions*			*Mortality*		
		Onset (min)	*No of animals*	*%*	*Onset (min)*	*No of animals*	*%*
Isoniazid	200	56 ± 4	20/20	100	64 ± 5	16/20	80
1c	20 + 200 Isoniazid	60 ± 3	12/14	86	62 ± 7	4/14[c]	28
1c	30 + 200 Isoniazid	51 ± 4	14/14	100	58 ± 3	12/14	86
1d	20 + 200 Isoniazid	61 ± 3	10/14	71	58 ± 5	4/14[c]	28
1d	30 + 200 Isoniazid	52 ± 2	14/14	100	53 ± 2	10/14	71
PTZ	60	3 ± 0.3	13/14	93			
1c	30 + 60 **PTZ**	4 ± 0.6	12/20	60			
1d	30 + 60 **PTZ**	2 ± 1.5	5/15[d]	33			

[a]Compounds **1c,d** (20–30 mg/kg) were administered ip 10 min after the subcutaneous administration of isoniazid (200 mg/kg). Values are the mean ± SEM of three separate experiments. [b]Compounds **1c,d** were administered ip 20 min before the injection of pentylenetetrazole (PTZ 60 mg/kg ip). Values are the mean ± SEM of three separate experiments. [c]$P < 0.025$ vs isoniazid-treated mice (Fisher's exact probability test). [d]$P < 0.005$ vs pentylenetetrazol-treated mice (Fisher's exact probability test).

also have a proconvulsant activity. Compound **2k** displayed both higher efficacy and higher potency than **2j**.

Compounds **1c,d** (20–30 mg/kg ip), which significantly decreased [^{35}S]TBPS binding in vitro, failed to antagonize (200 mg/kg sc) isoniazid-induced seizures in mice (table V), but at a dose of 30 mg/kg were able to antagonize the convulsions induced by pentylenetetrazole (PTZ, 65 mg/kg ip) (table V). Compound **1d** was more potent than **1c**.

The pharmacological data reported above are fully consistent with the profile predicted using a [^{35}S]-TBPS binding assay in vitro, and underscore the danger of using the GABA ratios to infer the likely functional effects of BZRs ligands. Our data therefore indicate that [^{35}S]TBPS binding is a more appropriate assay for this goal. Although some reports [8, 26] have already pointed out some discrepancies between in vivo and GABA ratios data, the latter in vitro assay is unfortunately still used extensively in structure–activity relationship study on BZRs ligands.

Molecular modeling studies

In an attempt to provide additional insight into the current SAR of BZRs ligands, we compared the key molecular features of high affinity compounds **1c,d** and **2j,k,q** with the known determinants for recognition and activation of BZRs. The most recent models have been proposed by the groups of Loew/Villar [8, 14] and Skolnick/Cook [11–13, 15, 16, 18, 19]. Loew/Villar's proposal was based on different orientation of the three putative anchoring sites (ie, center of an aromatic ring and two HB-accepting atoms) for agonists/antagonists and inverse agonists, whereas the Skolnick/Cook models for inverse agonists and antagonists presented an alternative alignment resulting from use of a different modeling approach (receptor mapping and 3-D QSAR) (fig 2).

At first glance the benzothiazole series of compounds **2j,k,q** show a structural analogy to the inverse agonist 3-carboxyethyl-β-carboline (β-CCE), as is clearly shown in the superposition of the two structures illustrated in figure 3. Since recent experimental evidence indicates that the active conformation of the ester moiety of β-carboline is *s-cis* [32], we have chosen this orientation for the overlay of the two ester ligands shown in figure 3. Following the hypothesis that nitrogen and carbonyl oxygen atoms engage simultaneously in a three-centered HB interaction with BZRs sites H1 [19], three structural elements, namely the centroid (Du2) between nitrogen (LP1)

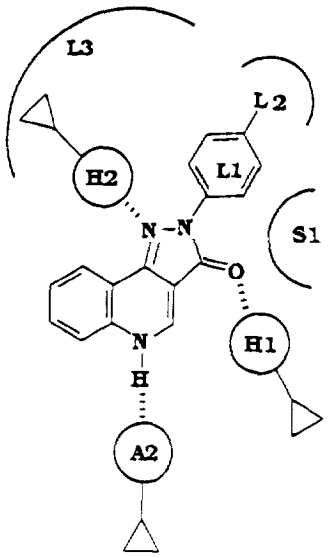


Fig 2. Proposed model [18, 19] of the BZR active site showing the main interactions of pyrazoloquinoline CGS 8216. Classical benzodiazepines, in addition to the interaction with the HB donor sites H1 and H2, may occupy the lipophilic area L3 with the 5-phenyl substituent. Inverse agonists, like β-carbolines, interact similarly with the H1 and H2 HB donor sites and make a further HB with the A2 HB acceptor site. Binding to H2 and A2 seems not to be necessary for inverse agonist activity (see ref [33] and the text).

and oxygen carbonyl (LP2) lone pairs, the centroid of the fused benzene ring A (Du1), and the ester CH_3 group, were used for the least-squares fit, leading to an rms value of 0.53 Å. The inverse agonist profile of **2j,k,q** is not fully interpretable with the Skolnick/Cook model for which the presence of a proton-donor group in the ligand (usually an NH group) is an important requirement for an inverse agonist activity. In this respect however, it is worthwhile remembering that the proton-donor ability of BZRs ligands has been found to increase potency (affinity) but is not a crucial prerequisite for an inverse agonist profile [33].

The possible explanation of the agonist effect of compounds **1c,d** is also far from obvious, due to the presence of multiple HB sites, particularly the two closely situated carbonyl groups which could facilitate alternative binding modes [34, 35]. Alignments a, c, and d (table VI and fig 4) reveal a generally poor fitting and furthermore imply the occupation of the sterically-forbidden region S1 by the carboethoxy, chlorine and thiazole moieties respectively. The most acceptable results come from the three- and four-point fittings according to alignments b, as summarized in table VII.

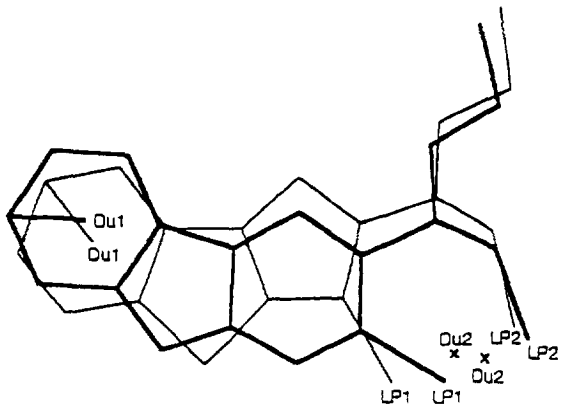


Fig 3. Three points superposition (LP1–LP2 centroid Du2, centroids Du1 and CH_3 carbon atoms) of β-CCE (bold line) and **2b** (thin line). Low energy conformers presenting an *s-cis* orientation of the NCC=O moiety have been chosen for fitting.

Alignments b1–b3 could be considered as the most satisfying ones, leading to acceptably low rms values and to sterically-allowed orientations. With these alignments, the terminal methyl group of the ethyl ester moiety of **1c** and **1d** occupies the receptor site L2 and this is consistent with their partial agonist profile activity.

Interestingly, another possible interpretation of the agonistic profile activity of compounds **1c,d** may be found by superimposing the diazepam molecule on CGS 9896 according to Fryer/Wermuth/Gardner (alignment 6c, page 213 of ref [19]) as reported in figure 5. In this way, substituents in position 6 of **1c,d** may lie on the same region (L3) occupied by the 5-phenyl ring of the potent agonist diazepam. In particular, the occurrence and strength of the interaction in the L3 area seem to depend upon the out-of-plane

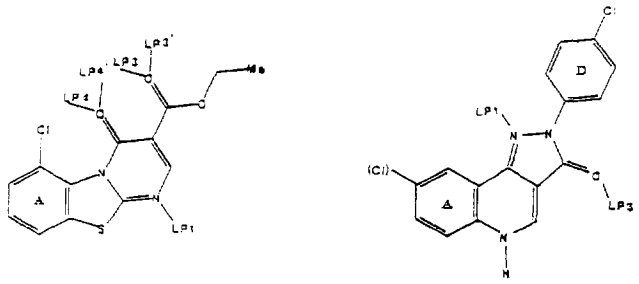


Fig 4. Pharmacophoric elements used for the alignments of **1d** and 8-Cl CGS 9896 (template) reported in figure 5 and tables VI and VII.

Table VI. Alternative alignments of **1d** vs 8-Cl CGS-9896[a].

Alignment	*Ring-A*	*Ring-D*	*LP1*	*LP3*	*4'-Cl*	*8-Cl*
a	Ring-A		LP1	LP4/LP4'/LP3/LP3'	CH_3	
b	Ring-A		LP4/LP4'/LP3/LP3'	LP1	CH_3	
c		Ring-A	LP1	LP4/LP4'/LP3/LP3'		CH_3
d		Ring-A	LP4/LP4'/LP3/LP3'	LP1	CH_3	

[a]For **1d**, two low-energy rotamers of the ester group with a C2–C3–C=O torsion angle of 180° (rotamer a) and 0° (rotamer b) have been used.

Table VII. Best rationalized alignments of **1d** vs CGS-9896[a].

Alignment	*Ring-A*	*LP1*	*LP3*	*4'-Cl*	*rms*[b] *(3p)*	*(4p)*
b1	Ring-A	LP4	LP1	CH_3	0.60 (0.60)	1.10 (1.15)
b2	Ring-A	LP4'	LP1	CH_3	0.89 (0.88)	1.09 (1.81)
b3	Ring-A	LP3	LP1	CH_3	1.07 (2.33)	1.17 (3.56)

[a]The rms (root mean squares deviations, Å) refer to three (3p)- or four-point (4p) fitting (left to right). [b]Data refer to rotamer b of **1d**; corresponding data of rotamer a of **1d** are reported in parentheses.

deformation of the otherwise planar ring system caused by the presence of a relatively rigid substituent in position 6 as also previously observed in other classes of BZRs ligands [17]. In figure 5 the overlay of **1d** on CGS-9896 according to the alignment b1 (four-point) has been reported. The diazepam molecule, fitted to CGS-9896 with the same criteria used for **1d**, has been added to show that 6-substituents may indeed contact the same lipophilic region (L3) occupied by the 5-phenyl ring of diazepam. In this model, the agonistic activity of **1c,d** may be ascribed to a possible simultaneous occupation of the L2 and L3 regions.

Conclusions

The results described herein have clearly shown that two pyrimidobenzothiazoles **1** and some imidazobenzothiazoles **2** possess diverse intrinsic efficacies and opposite pharmacological profiles which can be successfully predicted by [^{35}S]TBPS binding studies. In particular it was found that, while imidazobenzothiazoles **2j,k,q** possess inverse-agonist profiles, the pyrimidobenzothiazoles **1c,d** possess partial agonist properties. In this respect, recent progress in the pharmacology of central $GABA_A$ receptors has been characterized by evidence that BZRs ligands with low intrinsic efficacy (partial agonists) exhibit marked anxiolytic and anticonvulsant actions that are associated with reduced dependence and tolerance and with fewer unwanted side effects compared to classical agonists [36]. These data, together with recent preclinical studies have suggested potential therapeutical

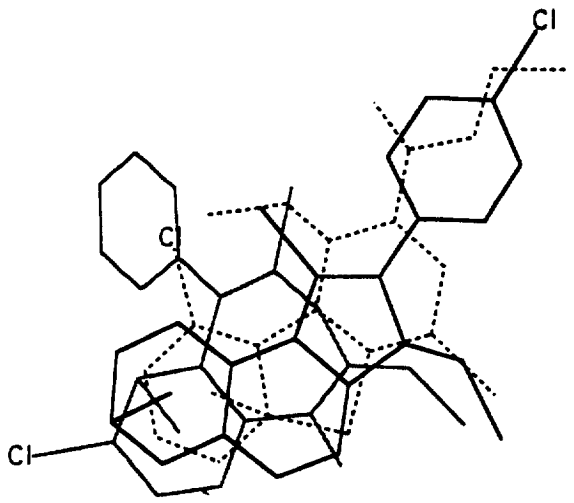


Fig 5. Overlay of **1d** and CGS 9896. The diazepam molecule (gray line) has been superimposed on CGS 9896 according to Fryer/Wermuth/Gardner (see text). The intersection of the 6-chlorine substituent of **1d** with the 5-phenyl ring of diazepam can be easily seen.

advantages of benzodiazepine receptors agonists with respect to full agonists. On the other hand, inverse agonists are potentially useful as cognitive-enhancer agents as recently observed for β-carbolines in passive avoidance tests in rats [37].

Finally, our paper pointed out a striking discrepancy between in vivo and GABA ratio data suggesting that structure–activity relationships study on BZRs ligands, in the absence of in vivo biology, can be more safely carried out employing [^{35}S]TBPS in vitro binding data. As far as the identification of determinants of recognition, common to all the BZ receptors, is concerned, our modeling studies are in agreement with the Skolnick/Cook model even though a different alignment of diazepam may suggest that an out-of-plane lipophilic region may be considered as a further pharmacophoric determinant of agonistic activity.

Experimental protocols

Chemistry

Melting points were determined in open capillary tubes with a Buchi apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer 283 spectrophotometer (KBr pellets). NMR spectra were determined on a Varian 390 or XL-200 or Bruker 300 MHz (NOESY experiment) instrument. ^{13}C NMR spectra were recorded on a Varian XL-200 instrument. Chemical shifts are given in δ values downfield from Me_4Si as internal standard. Mass spectra were recorded on a Hewlett-Packard 5995c GC-MS low resolution spectrometer. All compounds showed appropriate IR, ^{1}H NMR and mass spectra. Elemental analyses were carried out with a Carlo Erba model 1106 analyzer and results were within ±0.40% of the theoretical values. Silica gel 60 (Merck 70-230 mesh) was used for column chromatography.

*General procedure for the preparation of 2-alkoxycarbonyl imidazo[2,1-b]benzothiazoles **2e–p** and thiazoles **8b,c***

Ethyl bromopyruvate (20 mmol) was added dropwise to a stirred solution of the appropriate 2-aminobenzothiazole (15 mmol) in dry THF (20 mL). The mixture was stirred for 4 h, then EtOH (10 mL) was added and the resulting solution was refluxed for at least 3 h. The progress of reaction was monitored by TLC. Solvent was evaporated under reduced pressure and the residue was dissolved in $CHCl_3$ (20 mL) and washed with 5% $NaHCO_3$. Evaporation of the solvent gave a residue which was purified by silica-gel column chromatography (light petroleum ether/ethyl acetate as eluent). The procedure was partially modified as follows when esters **2i,n** were prepared: the appropriate 2-aminobenzothiazole **4i** or **4n** (26 mmol) and ethyl bromopyruvate (34 mmol) were allowed to react in refluxing DMF (30 mL) for 3 h. Evaporation of the solvent gave a residue which was purified by column chromatography (light petroleum ether/ethyl acetate 7:3 v/v as eluent).

6-Ethoxycarbonyl-imidazo[2,1-*b*]thiazoles **8b,c** were prepared in the same way starting from the appropriate 2-aminothiazole.

Spectral data for compounds **2e–r** are as follows: **2e**: IR 1740 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.43 (t, J = 6.0 Hz, 3H, CH_3), 4.43 (q, J = 6.0 Hz, 2H, OCH_2), 7.06–7.73 (m, 3H, arom), 8.36 (s, 1H, C(3)-H); MS, *m/z* 264 (31, M$^+$), 192 (base). Anal $C_{12}H_9FN_2O_2S$ (C, H, N). **2h**: IR 1700 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.43 (t, J = 6.0 Hz, 3H, CH_3), 3.86 (s, 3H, OCH_3), 4.43 (q, J = 6.0 Hz, 2H, OCH_2), 6.93–7.60 (m, 3H, arom), 8.30 (s, 1H, C(3)-H); MS, *m/z* 276 (40, M$^+$), 204 (base). Anal $C_{13}H_{12}N_2O_3S$ (C, H, N). **2i**: IR 1710 cm^{-1}; ^{1}H NMR (DMSO-d_6), δ: 1.33 (t, J = 6.0 Hz, 3H, CH_3), 4.33 (q, J = 6.0 Hz, 2H, OCH_2), 8.30 (s, 1H, arom), 8.43 (m, 1H, arom), 9.16 (m, 2H, arom + C(3)-H); MS, *m/z* 291 (35, M$^+$), 219 (base). Anal $C_{12}H_9N_3O_4S$ (C, H, N). **2k**: IR (KBr) 1730 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.43 (t, J = 6.0 Hz, 3H, CH_3), 4.03 (s, 3H, OCH_3), 4.43 (q, J = 6.0 Hz, 2H, OCH_2), 6.83–7.00 (m, 1H, arom), 7.16–7.30 (m, 2H, arom), 8.56 (s, 1H, C(3)-H); MS, *m/z* 276 (60, M$^+$), 204 (base). Anal $C_{13}H_{12}N_2O_3S$ (C, H, N). **2l**: IR 1730 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.43 (t, J = 6.0 Hz, 3H, CH_3), 2.76 (s, 3H, CH_3), 4.43 (q, J = 6.0 Hz, 2H, OCH_2), 7.20–7.63 (m, 3H, Arom), 8.50 (s, 1H, C(3)-H); MS, *m/z* 260 (40, M$^+$), 188 (base). Anal $C_{13}H_{12}N_2O_2S$ (C, H, N).

For the sake of comparison, ^{13}C NMR data of low field resonating quaternary and tertiary carbons of selected compounds **2j,l** are reported together with those of the 5-unsubstituted parent compound **2b**.

2j: Quaternary C 162.5, 148.2, 138.2, 132.8, 128.9, 120.5; tertiary C 127.5, 122.8, 122.5, 120.2. **2l**: Quaternary C 162.56, 148.7, 138.4, 130.9, 124.8; tertiary C 128.5, 125.6, 122.0, 119.4. **2b**: Quaternary C 162.3, 148.5, 138.3, 131.4, 130.9; tertiary C 126.6, 126.1, 124.6, 116.6, 113.6. **2n**: IR 1730 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.34 (t, J = 6.0 Hz, 3H, CH_3), 4.43 (q, J = 6.0 Hz, 2H, OCH_2), 7.46 (d, J = 1 Hz, 1H, arom), 7.60 (d, J = 1 Hz,1H, arom), 8.83 (s, 1H, C(3)-H); MS, *m/z* 314 (38, M$^+$), 242 (base). Anal $C_{12}H_8Cl_2N_2O_2S$ (C, H, N). **2o**: IR 1710 cm^{-1}; ^{1}H NMR (CF_3COOD), δ: 1.53 (t, J = 6.0 Hz, 3H, CH_3), 4.63 (q, J = 6.0 Hz, 2H, OCH_2), 8.13 (s, 1H, arom), 8.30 (s, 1H, arom), 8.86 (s, 1H, C(3)-H); MS, *m/z* 314 (35, M$^+$), 242 (base). Anal $C_{12}H_8Cl_2N_2O_2S$ (C, H, N). **2p**: IR 1730 cm^{-1}; ^{1}H NMR (CF_3COOD), δ: 1.56 (t, J = 6.0 Hz, 3H, CH_3), 4.66 (q, J = 6.0 Hz, 2H, OCH_2), 7.97 (d, J = 9.0 Hz, 1H, arom), 8.13 (d, J = 9.0 Hz, 1H, arom), 8.93 (s, 1H, C(3)-H); MS, *m/z* 314 (34, M$^+$), 242 (base). Anal $C_{12}H_9Cl_2N_2O_2S$ (C, H, N).

Spectral data for compounds **8b,c** are as follows: **8b**: IR 1730 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.43 (t, J = 6.0 Hz, 3H, CH_3), 4.50 (q, J = 6.0 Hz, 2H, OCH_2), 6.90 (s, 1H, =CH), 7.50–7.70 (m, 5H, arom), 8.26 (s, 1H, C(3)-H); MS, *m/z* 272 (31, M$^+$), 200 (base). Anal $C_{14}H_{12}N_2O_2S$ (C, H, N). **8c**: IR 1740 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.43 (t, J = 6.0 Hz, 3H, CH_3), 4.40 (q, J = 6.0 Hz, 2H, OCH_2), 6.90 (s, 1H, =CH), 7.50–7.55 (m, 4H, arom), 8.20 (s, 1H, C(3)-H); MS, *m/z* 306 (47, M$^+$), 234 (base). Anal $C_{10}H_{11}ClN_2O_2S$ (C, H, N).

*Methyl 5-chloroimidazo[2,1-b]benzothiazole-2-carboxylate **2q**.* A solution of **2j** (8 mmol) in 1 N NaOH (20 mL) and EtOH (10 mL) was heated on a steam bath for 1 h. The solvent was evaporated under reduced pressure and the resulting residue dissolved in water. Treatment with aqueous HCl gave the crude carboxylic acid which was treated with boron trifluoride etherate (6 mmol) and methanol (12 mL). This mixture was refluxed for 24 h. The solvent was then removed under reduced pressure and the residue was dissolved in $CHCl_3$ (50 mL). The organic solution was washed with 10% Na_2CO_3, dried (Na_2SO_4) and evaporated. The resulting residue was purified by column chromatography (light petroleum ether/ethyl acetate 8:2 v/v as eluent) to give **2q** (0.84 g, 50% yield). IR 1740 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 3.96 (s, 3H, CH_3), 7.20–7.63 (m,

3H, arom), 8.86 (s, 1H, C(3)-H); MS, *m/z* 266 (83, M^+), 208 (base). Anal $C_{11}H_7ClN_2O_2S$ (C, H, N).

Benzyl 5-chloroimidazo[2,1-b]benzothiazole-2-carboxylate **2r**. To a mixture of the crude carboxylic acid obtained as above starting from **2j** (9 mmol) in dry toluene (30 mL), oxalyl chloride (14 mmol) was added dropwise . The mixture was stirred at room temperature for 18 h, and then filtered to give the acid chloride of **2j**. A mixture of this compound (2.2 g, 8 mmol) and HCl-saturated benzyl alcohol (20 mL) was refluxed for 2 h, washed with 5% Na_2CO_3 and evaporated to give a residue which was purified by column chromatography (light petroleum ether/ethyl acetate 97:3 v/v as eluent) affording **2r** (0.7 g, 22% yield). IR 1650 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 5.43 (s, 2H, CH_2), 7.23–7.66 (m, 8H, arom), 8.90 (s, 1H, C(3)-H); MS, *m/z* 342 (17, M^+), 208 (base). Anal $C_{17}H_{11}ClN_2O_2S$ (C, H, N).

Imidazo[2,1-b]benzothiazole-2-carboxyhydrazide **2t**. A mixture of **2b** (1.3 g, 5.3 mmol) and hydrazine hydrate 90% (20 mL) was stirred overnight at room temperature. The precipitate collected by filtration gave the pure compound **2t** (0.4 g, 33% yield). IR 3340, 3120, 1650 cm^{-1}; ^1H NMR (CH_3COOD) δ: 7.25–7.85 (m, 4H, arom), 8.60 (s, 1H, C(3)-H); MS, *m/z* 232 (44, M^+), 201 (base). Hydrazide **2u** was prepared in the same way starting from **2j**. Anal $C_{10}H_8N_4OS$ (C, H, N).

1,3,4-Triazolo[2,1-b]benzothiazole **7a**. A mixture of 2-hydrazinobenzothiazole (1.6 g, 9.7 mmol), trimethyl orthoformate (10.3 g, 97 mmol) and silica gel (1 g) in xylene (30 mL) was refluxed for 3 h. The solvent was evaporated under reduced pressure and the resulting residue extracted with $CHCl_3$ (3 x 50 mL). Evaporation of the solvent to dryness gave a residue which was purified by column chromatography (light petroleum ether/ethyl acetate 7:3 v/v as eluent) to give **7a** (0.5 g, 29% yield). IR 3100, 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.40–7.60 (m, 2H, arom), 7.76–7.83 (m, 2H, arom), 9.03 (s, 1H, C(3)-H); MS, *m/z* 175 (97, M^+), 148 (base). Anal $C_8H_5N_3OS$ (C, H, N).

3-Phenyl-1,2,4-triazolo[2,1-b]benzothiazole **7b**. A mixture of 2-hydrazino benzothiazole (0.9 g, 55 mmol), trimethylorthobenzoate (10 g, 55 mmol), xylene (20 mL) and catalytic amount of *p*-toluenesulfonic acid was refluxed for 3.5 h. The solvent was evaporated under reduced pressure and the resulting residue was purified by column chromatography (light petroleum ether/ethyl acetate 7:3 v/v as eluent) to give **7b** (0.5 g, 37% yield). IR 3040, 1550 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.26–7.86 (m, 9H, arom); MS, *m/z* 251 (96, M^+), 148 (base). Anal $C_{14}H_9N_3S$ (C, H, N).

Biological methods

Radioligand binding assays

Ligand binding to rat brain. [^{3}H]flunitrazepam (New England Nuclear, Boston, USA) had a specific activity of 84.3 Ci/ mmol and a radiochemical purity >74.6%. Sprague–Dawley rats (150–200 g) were killed by decapitation and whole brains (excluding cerebellum and pons medulla) were quickly removed. The brains were homogenized in 20 volumes of ice-cold 0.32 M sucrose with a Potter. The homogenate was centrifuged for 5 min at 2000 *g* at 4 °C and the supernatant was centrifuged for 10 min at 40 000 *g* at 4 °C. The pellet was suspended in 30 mL of 50 mM Tris-HCl cold buffer, pH 7.4, and centrifuged for 30 min at 40 000 *g* at 4 °C. This pellet was suspended in 8–10 mL of Tris-HCl buffer. Protein content was determined by the Lowry method [38] using bovine serum albumin as standard. BZR binding activity was determined as follows: 50 µL of membrane suspension were incubated in quadruplicate with 0.67 nM [^{3}H]flunitrazepam and a 40 µM inhibitor for 90 min at 4 °C in 50 mM Tris-HCl cold buffer (500 µL final volume). After this incubation time the samples were diluted with 5 mL of Tris-HCl cold buffer and immediately filtered under reduced pressure through glass-fiber filter disks (Whatman, GF/C), followed by washing with 5 mL of the same cold buffer, and counted in 4 mL of Ready Protein Beckman liquid scintillation cocktail. The imidazobenzothiazole derivatives **2** were dissolved in 50% ethanol/Tris-HCl buffer and added to the assay mixture. Non-specific binding was determined by incubating membranes and [^{3}H]flunitrazepam in the presence of 10 µM diazepam. Specific binding was obtained by subtracting non-specific binding from total binding and was approximately 85–90% of the total binding. Six to eight concentrations of the compounds in quadruplicate were added to samples to determine IC_{50} values in the absence and in the presence of 10 µM GABA in parallel experiments. Values shown for the GABA ratio are means of the ratio of IC_{50} without GABA to IC_{50} with 10 µM GABA in three independent experiments.

Ligand binding to mouse cortical membranes. Male CD-1 mice (Charles River, Como, Italy) with body weights 25–30 g were kept under a 12 h light/dark cycle at a temperature of 23 ± 2 °C and 65% humidity. Upon arrival at the animal facilities there was a minimum of seven days' acclimatization, during which the animals had free access to food and water. The animals were sacrificed by cervical dislocation in the middle of the light phase. The brains were rapidly removed, the cerebral cortex was dissected out and was used for the measurement of [^{3}H]flunitrazepam binding, [^{35}S]TBPS binding, and $^{36}Cl^-$ uptake.

[^{3}H]flunitrazepam binding. Cerebral cortices were homogenized in 10 volumes of ice-cold distilled H_2O with a polytron PT 10 (setting 5, for 20 s) and centrifuged at 48 000 *g* for 10 min. The pellet was reconstituted in 50 volumes of 50 mM Tris-HCl buffer (pH 7.4). Aliquots of 100 µL tissue homogenate (200–300 µg of protein) were incubated in the presence of [^{3}H]flunitrazepam at a final concentration of 0.5 nM, in a total incubation volume of 500 µL. The compounds were dissolved in dimethylsulfoxide and serial dilutions were made up in buffer and added in 50 µL aliquots. After 60 min incubation at 4 °C, the assay was terminated by rapid filtration through glass-fiber filter strips (Whatman GF/B). The filters were rinsed with 2 x 4 mL ice-cold 50 mM Tris-HCl buffer with a cell Harvester filtration manifold (Model M-24, Brandel) and transferred in plastic minivials with 3 mL scintillation fluid (Atomlight, New England Nuclear). Non-specific binding was determined as binding in the presence of 5 µM diazepam.

[^{35}S]TBPS Binding. Cerebral cortices were homogenized with a polytron PT 10 (setting 5, for 20 s) in 50 volumes of ice-cold 50 mM Tris-citrate buffer (pH 7.4 at 25 °C) containing 100 mM NaCl. The homogenate was centrifuged at 20 000 *g* for 20 min and reconstituted in 50 volumes of 50 mM Tris-citrate buffer without NaCl. [^{35}S]TBPS binding was determined in a final volume of 500 µL, consisting of 200 µL tissue homogenate (200–300 µg protein), 50 µl 2 nM [^{35}S]TBPS, 50 µL 0.2 M NaCl, 50 µL drugs, dissolved as describe above, or solvent (total and non-specific samples). The incubations (25 °C) were started by the addition of tissue homogenate and were terminated 90 min later by rapid filtration through glass-

fiber filter strips (Whatman GF/B) with a filtration manifold (Model M24, Brandel). The filters were rinsed with 2 x 4 mL ice-cold 50 mM Tris-citrate buffer. Non-specific binding was defined as binding in the presence of 100 μM picrotoxin, and represented about 10% of total binding.

$^{36}Cl^-$ uptake. Membrane vesicles from mouse cerebral cortices were prepared according to Harris and Allan [39] with minor modifications. Cerebral cortices were homogenized by hand (10–12 strokes) in 10 volumes (w/v) of ice-cold buffer (in millimol: NaCl, 145; KCl, 5; $MgCl_2$, 1; D-Glucose, 10; $CaCl_2$, 1; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10, adjusted to pH 7.5 with Tris base) with a glass–glass homogenizer. The homogenate was centrifuged at 1000 *g* for 15 min at 4 °C, the supernatant was discarded and the pellet was resuspended in the same volume of buffer and recentrifuged at 1000 *g* for 15 min at 4 °C. The final pellet was gently resuspended in the same buffer to a final protein concentration of 7–8 mg/mL. Aliquots of membranes (200 μL) were preincubated in a shaking water bath at 30 °C for 10 min. $^{36}Cl^-$ uptake was initiated by the addition of 200 μL $^{36}Cl^-$ 1m Ci/mL) or a solution of $^{36}Cl^-$ and muscimol (5 μM). Drugs (2 μL dissolved in dimethylsulfoxide) were preincubated with membrane vesicles at 30 °C for 10 min. Three seconds after the addition of $^{36}Cl^-$, uptake was terminated by the addition of ice-cold buffer (2 x 4 mL) followed by rapid vacuum filtration (20 inches of Hg) through glass fiber filters, Whatman GF/C (presoaked with 0.05% polyethyleneimine to reduce non-specific binding of $^{36}Cl^-$) using a Hoefer manifold (Hoefer Scientific, San Francisco, CA). The filters were washed with ice-cold buffer (10 x 1 mL) and the $^{36}Cl^-$ content of the filters was determined by liquid scintillation counting. The amount of $^{36}Cl^-$ bound to the filters in the absence of membranes was subtracted from all values. Filter-bound radioactivity was quantitated by liquid scintillation spectrometry. Protein concentration was assayed by the method of Lowry [38] with bovine serum as standard. Biochemical data were analyzed using the Student's *t* test and IC_{50} values were determined from displacement curves with the Ligand program [40].

In vivo studies

Mice treatment. The compounds **2j,k,q** and flumazenil were suspended in distilled water with a drop of Tween 80 per 5 mL, sonicated and injected ip (10 mL per kilogram of body mass). Isoniazid and pentylenetetrazole (PTZ) were dissolved in distilled water and administered sc or ip (10 mL/kg) respectively. **2j** and **2k** were injected 45 min after isoniazid, while **1c** and **1d** were injected 10 min after isoniazid and 20 min before PTZ respectively. Flumazenil was administered 15 min before **2q** and 10 min before **1c**. After the drug administration, the time of onset of seizure activity and the pattern of the seizures were recorded.

Behavioral data were analyzed utilizing Fisher's exact probability test and the Student's *t* test.

Molecular modeling

The present studies were performed on an Evans & Sutherland PS 390 graphics workstation networked to a Digital VAX 3100 using the molecular modeling software SYBYL version 5.41 (Tripos Associates, Saint Louis, MO). Molecular models were constructed using standard bond distances and angles within SYBYL and fully optimized with AM1 (QCPE 506). The ester side chains were optimized holding the heterocyclic core structure fixed and using a 30° increment for the ring–carbonyl torsion angles in the range of 0–360°. The conformations chosen for the fitting in figures 3 and 5 were within 0.5 kcal of the minimum energy conformer. The FIT and MVOLUME SYBYL commands were used for the least square fitting and volume analysis respectively. Calculations of ring centroids, interatomic distances, and angles were carried out using the graphic capability of the software. The hydrogen bond extension vectors (HBVs) were generated starting with SYBYL-generated lone pairs of electrons, and adjusting the heteroatom–lone pair distance to 1.84 Å and the C–N–HBV and C=O–HBV valence angles to 120° and 135° respectively, in order to mimic ideal hydrogen bond geometries observed in X-ray crystallographic structures [41–45].

Acknowledgments

The authors wish to thank K Koehler (IRBM, Pomezia, Rome) for helpful comments and suggestions. This work was supported by a grant from MURST and CNR, Rome.

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