



Use of imidazo[1,5-*a*]quinoline scaffold as the pharmacophore in the design of bivalent ligands of central benzodiazepine receptors

Marco Paolino^{a,*}, Mario Saletti^a, Jacopo Venditti^a, Federica Castriconi^a, Germano Giuliani^a, Samuele Maramai^a, Alessandra Toti^b, Carla Ghelardini^b, Rosanna Matucci^b, Nancy Alcazar Villalobos^c, Maurizio Anzini^{a,1}, Andrea Cappelli^a

^a Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Via A. Moro 2, 53100 Siena, Italy

^b Dipartimento di Neuroscienze, Psicologia, Area del Farmaco e Salute del Bambino (NEUROFARBA) – Sez. Farmacologia e Tossicologia, Università di Firenze, Viale G. Pieraccini 6, 50139 Firenze, Italy

^c Medicines Discovery Institute, Cardiff University, Park Place, CF10 3AT Cardiff, Wales, United Kingdom

ABSTRACT

The imidazo[1,5-*a*]quinoline scaffold of central benzodiazepine receptor (CBR) ligands was used as the pharmacophore in the design of bivalent ligands bearing spacers showing variable length and different physicochemical features. The newly designed compounds were synthesized along with the corresponding reference monovalent compounds bearing the corresponding spacers terminated with a *tert*-butoxycarbonyl group. The novel compounds were tested in binding assays with different CBR preparations such as the cerebral cortex from male CD-1 albino mice or the human recombinant $\alpha 1\beta 3\gamma 2$ and $\alpha 2\beta 3\gamma 2$ γ -aminobutyric acid type A receptors (GABA_ARs) stably expressed in mouse L(tk-) cells. The tested compounds showed IC₅₀ values from the sub-micromolar up to the nanomolar range with very similar inhibition constants values for the two isoforms of GABA_ARs. The similarity in the affinity between the bivalent ligands and the corresponding monovalent ones appeared to rule out any bivalent interactions of these ligands with the two isoforms of GABA_ARs. Similarly, both series were able to inhibit the binding of radiolabeled flumazenil to GABA_ARs in cortical membranes of albino CD-1 mice, but most of the tested compounds showed biphasic inhibition curves, suggesting the existence of two well-distinct populations of binding sites. Finally, some CBR ligands selected from the bivalent ligands (i.e. **6a,c**) and from the reference monovalent ligands (i.e. **7a**) were then tested *in vivo* for their potential pharmacological effects, evaluating four classical benzodiazepine actions such as anticonvulsant, anxiolytic, locomotor, and anti-amnesic activities. All the tested compounds showed anticonvulsant and anxiolytic properties with neither muscle relaxant effect nor learning and memory impairments.

1. Introduction

The central neurotransmitter γ -aminobutyric acid (GABA) exerts its action through both GABA type A (GABA_ARs) and type B (GABA_BRs) receptors, modulating the excitability of many central nervous system (CNS) pathways.¹ GABA_ARs are chloride ions channel belonging to ligand-gated ion channels (LGICs) of the Cys-loop superfamily, to which nicotinic acetylcholine, glycine, zinc-activated, and 5-HT₃ receptors also belong. Cys-loop receptors are targets of many drugs and feature the five-subunit assembly forming pentameric arrangements around a central ion-conducting pore.¹ The GABA_AR function is regulated by allosteric sites interacting with a large diversity of agents in addition to the agonist binding site.² The most known positive modulators of the GABA_ARs is the classical 1,4-benzodiazepine diazepam (**1**, Figure 1).

The positive allosteric modulators (PAMs) of GABA_ARs are an

important class of drugs used as sedatives, anticonvulsants, anxiolytics, and muscle relaxants. On the other hand, negative GABA_ARs modulators (i.e. inverse agonists) show anxiogenic and convulsant effects.^{3–6} Finally, neutral modulators, [i.e. the imidazo[1,5-*a*][1,4]benzodiazepines flumazenil (**2**, Figure 1)] interact with GABA_ARs but do not show any functional efficacy. Therefore, flumazenil is considered to antagonize the activity of both positive and negative GABA_ARs modulators acting *via* the central benzodiazepine receptor (CBR). Positive modulators are documented as molecules with amnesic effects in animal and man,^{7–10} whereas negative modulators were supposed to possess pro-cognitive properties.^{11,12} Unfortunately, the employment of CBR inverse agonists in the treatment of neurological disorders connected with cognitive impairment was limited by their anxiogenic and convulsant effects.¹³

Even if a large variety of different subunits (i.e. $\alpha 1-6$, $\beta 1-4$, $\gamma 1-4$, δ , ϵ , π ,

* Corresponding author.

E-mail address: paolino3@unisi.it (M. Paolino).

¹ Present address, Via del bosco 19, 53019 Castelnuovo Berardenga, Italy.

θ and ρ_{1-3}) was cloned and sequenced, most of GABA_ARs are constituted by the combination of α , β , and γ -subunits organized in a 2:2:1 stoichiometry.¹⁴ Among the possible combinations stemming from the co-assembly of the subunits, barely the receptors composed by a γ_2 or γ_3 ¹⁵ subunit combined with α_1 , α_2 , α_3 , or α_5 ones appear to bind CBR ligands with noteworthy affinity, and the binding domain is recognized to be located at the interface between α and γ subunits.¹⁶ Molecular genetics and pharmacological investigations suggested for α_1 subunit an important role in the sedative and muscle relaxant effects of the non-selective CBR agonists, while α_2 or α_3 subunits are associated with anxiolytic and anticonvulsant effects.¹⁷⁻¹⁹ The recognition of the physiological and pharmacological roles of α subunits in GABA_AR functions has renewed the general interest with the assumption that new drugs could be obtained with fewer side-effects or different therapeutic uses with respect to the classical benzodiazepines.²⁰⁻²⁷ Many CBR ligands have been developed showing different GABA_AR subtype selectivity: selective binding (i.e. by establishing a receptor-ligand complex with a particular receptor subtype) or by selective efficacy (i.e. by obtaining a biological response after binding to the receptor).^{20,28}

Interestingly, imidazo[1,5-*a*][1,4]benzodiazepine derivatives **3** have been reported to show the full range of intrinsic efficacy, which was modulated in a rather subtle manner by the substitution pattern,^{29,30} and similar results were obtained when the seven-membered ring of the benzodiazepine system was contracted as in the series of imidazo[1,5-*a*]quinoxaline derivatives **4**.³¹⁻³⁶ Moreover, the work made by the Upjohn researchers suggested the existence of a low affinity-binding site on GABA_ARs,³⁷ and the structure of imidazo[1,5-*a*]quinoxaline derivatives **4** was easily translated into the imidazo[1,5-*a*]quinoline one of **5** (see

Figure 1). Compound **5a** was identified as a drug candidate for the treatment of anxiety, but its development was discontinued for safety reason.³⁸

Intriguingly, the structure-activity relationship (SAR) analysis suggested that the presence of bulky substituents was tolerated by CBR binding site when they are located in the ligand region corresponding to positions 4 and 5 of the imidazo[1,5-*a*]quinoline nucleus consistently in agreement with that we observed with our 5-HT₃ receptor ligands based on quinoline structure.^{39,40}

The structural analogies between GABA_A and 5-HT₃ receptors stimulated the application of our design approach previously used in studying 5-HT₃ receptors and leading to the discovery of multivalent ligands.^{41,42} Intriguingly, the docking studies performed on a 5-HT₃ receptor model allowed us to ascertain the existence of several potential accessory binding sites. Thus, we assumed that multivalency in 5-HT₃ receptor could involve receptor domains different from the main binding site.⁴¹ A similar information was not available for GABA_ARs. Thus, in the first part of this project, we performed a careful exploration of the SAR of imidazo[1,5-*a*]quinoline derivatives **5** in the interaction with CBR.⁴³ The results of such study allowed us to identify a suitable pharmacophore candidate, which was employed in the second part described in the present paper, for the design of bivalent CBR ligands **6a-e** (Figure 2).

In particular, our approach consisted in the design of bivalent ligands **6a-e** and the corresponding reference monovalent ligands **7a-e**, in which the second imidazo[1,5-*a*]quinoline pharmacophore is replaced by a *tert*-butoxy group. In our approach, reference monovalent and bivalent ligands were designed to directly compare binding affinity data and to

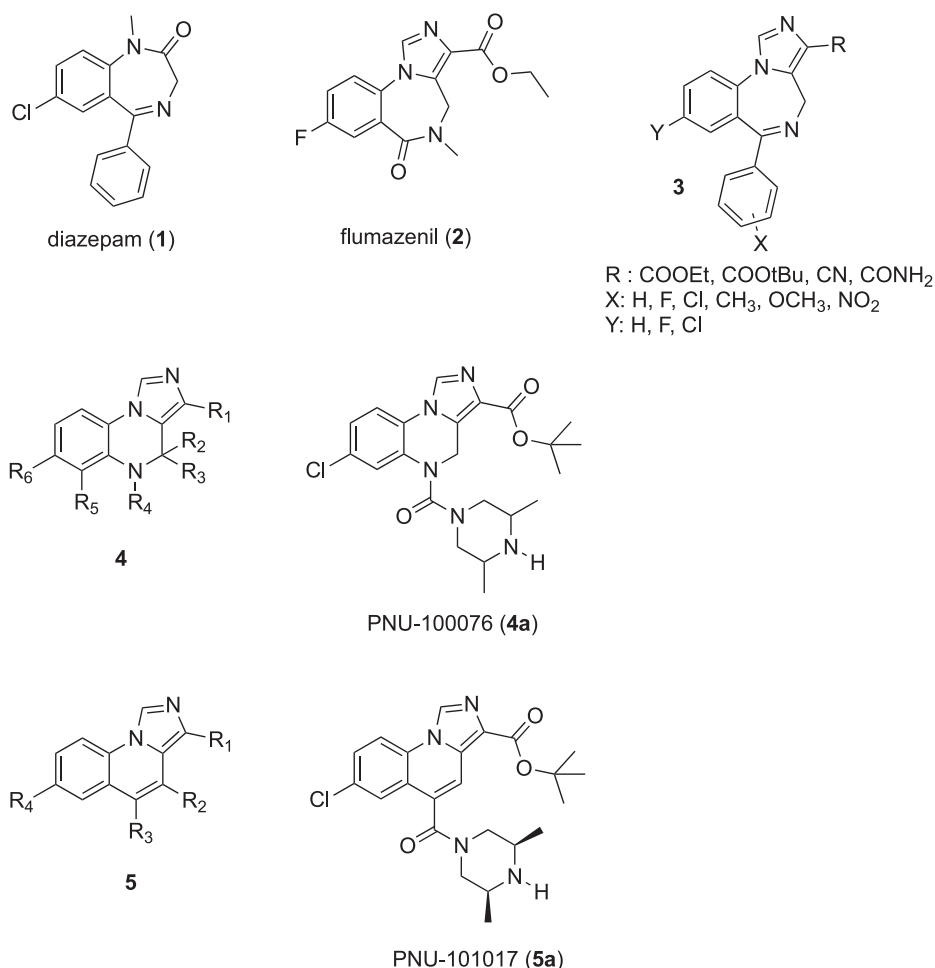


Fig. 1. Structure of reference CBR ligands showing different scaffolds.

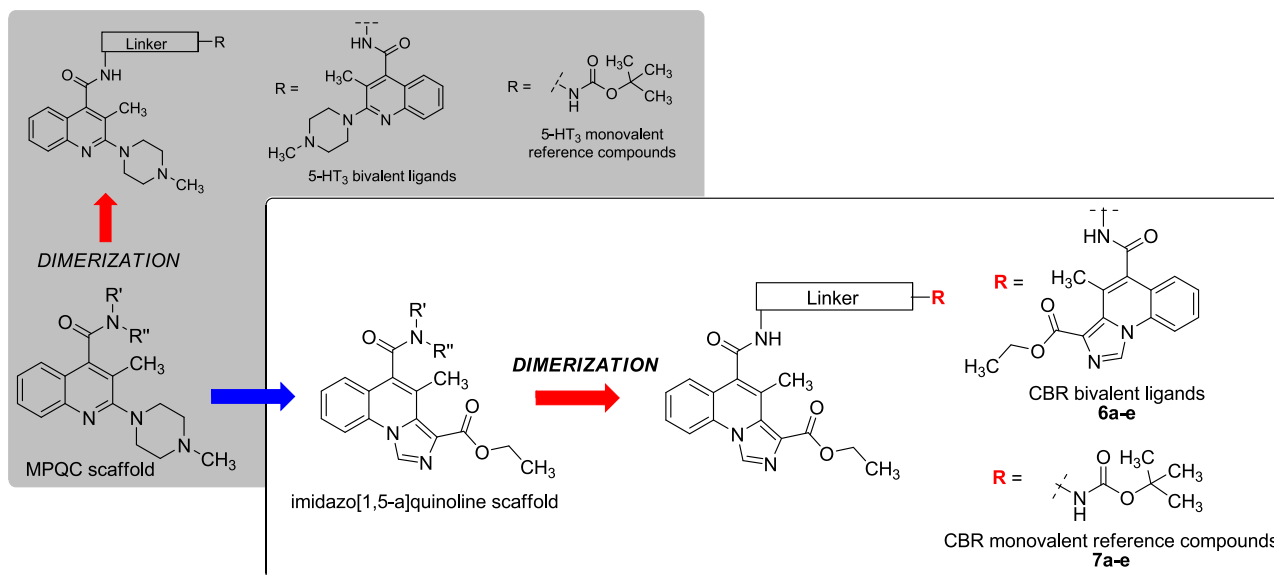


Fig. 2. Application of the imidazo[1,5-a]quinoline scaffold in the design of bivalent and monovalent CBR ligands **6a-e** and **7a-e**.

properly evaluate the multivalent approach.^{41,42,44-46} In particular, the bivalent interaction of a bivalent ligand should be able to produce an increase in the binding affinity with respect to that shown by the corresponding monovalent one. These newly designed compounds were synthesized by means of the chemistry previously developed in our laboratories (see [Supporting Information](#)) and were evaluated in binding assays by using different CBR preparations (i. e. in cerebral cortex from male CD-1 albino mice or in mouse L(tk-) cells stably expressing human recombinant $\alpha 1\beta 3\gamma 2$ and $\alpha 2\beta 3\gamma 2$ GABA_ARs) and by using different experimental set-up (see below). Moreover, a few selected CBR ligands (**6a,c** and **7a**) were then tested *in vivo* for their potential pharmacological effects, by taking into consideration four benzodiazepine actions such as anticonvulsant, anxiolytic, locomotor, and anti-amnesic

activities.

2. Results and discussion

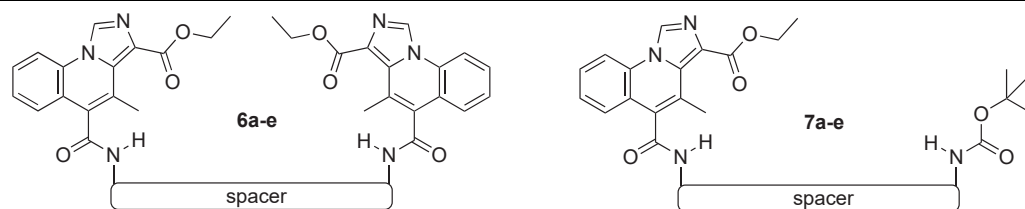
2.1. *In vitro* binding

The affinity of bivalent ligands **6a-e** and the corresponding reference monovalent ligands **7a-e** for CBR in cortical membranes of albino CD-1 mice was measured by means of competition experiments against the radiolabeled antagonist [³H]flumazenil and the results of the binding studies are summarized in [Table 1](#).

The most surprising result was the rather peculiar behaviour of most of the tested compounds in the test system used in this preliminary

Table 1

Inhibition of [³H]flumazenil specific binding to CBR in cerebral cortex from male CD-1 albino mice of bivalent ligands **6a-f** and reference monovalent ligands **7a-e**.^a



Compd	Spacer	Methylene equivalents	<i>K_i</i> (nM)	<i>K_i</i> LAS (nM)	<i>K_i</i> HAS (nM)
			Monophasic curve	Biphasic curve	Biphasic curve
6a	-(CH ₂) ₆ -	6		60	0.022
6b	-(CH ₂) ₇ -	7		12	0.00068
6c	-(CH ₂) ₈ -	8		52	0.050
6d	-(CH ₂ -CH ₂ -O) ₂ -CH ₂ -CH ₂ -	8	18		
6e	-(CH ₂ -CH ₂ -O) ₇ -CH ₂ -CH ₂ -	23	41		
7a	-(CH ₂) ₆ -	6		35	0.000065
7b	-(CH ₂) ₇ -	7		6.4	0.000061
7c	-(CH ₂) ₈ -	8		51	0.0050
7d	-(CH ₂ -CH ₂ -O) ₂ -CH ₂ -CH ₂ -	8	12		
7e	-(CH ₂ -CH ₂ -O) ₇ -CH ₂ -CH ₂ -	23	0.66		
1				3.5	0.0000049
2				1.3	0.0000029

^a Brain cortex membranes were incubated with 0.2 nM [³H]flumazenil ([³H]Ro 15-1788) in the absence and presence of increasing concentrations of the indicated compounds. All incubations were performed for 90 min at 0 °C (i. e. in ice) in the assay buffer. Non-specific binding was determined in the presence of 10 μM of diazepam. Concentration-response curves were analyzed using the curve-fitting program GraphPad Prism. Ten concentrations of displacers were examined each in duplicate. Each value was the average of 4-6 independent experiments. The *K_i* values were calculated from the IC₅₀ by the method of Cheng and Prusoff using the *K_d* value (0.47 nM) obtained for [³H]flumazenil.

biological evaluation. In fact, most of the tested compounds showed biphasic inhibition curves suggesting the existence of two well-distinct populations of binding sites. Even more surprising was the apparent affinity of some of the tested compound for the high affinity site, which was estimated to be in the sub-picomolar range. The most potent ligands in the interaction with the high affinity binding site were the reference monovalent ligands **7a-c** and the corresponding bivalent ligands **6a-c**, with reasonable affinity modulations in relation to the structural variations. For instance, the heptamethylene spacer was optimal for the binding potency in both the series **6a-c** and **7a-c** as previously observed in the bivalent ligands for 5HT₃ receptors.⁴¹ On the other hand, the affinity of these compounds for the low affinity site was markedly lower with *K_i* values spanning from the sub-micromolar up to the nanomolar range, with stringent analogies with the values displayed by the ligands showing monophasic inhibition curves. The structure-affinity relationship analysis failed in revealing evidence of bivalent interactions of these compounds at CBR binding site since the bivalent ligands **6a-e** appeared to be less potent than the corresponding reference monovalent ligands **7a-e**, in which the second imidazo[1,5-*a*]quinoline pharmacophore is replaced by a *tert*-butoxy group.

A large body of evidence reported in the literature supports the existence of a biphasic binding at CBRs and of very high affinity sites.⁴⁷⁻⁵² The intriguing work performed by Metha and Shank⁵² took into consideration the possible role of several parameters of the experimental set-up (i.e. brain areas, incubation temperature and time, radioligand etc.) in the biphasic binding. Thus, the results obtained in albino CD-1 mice required to be challenged in a different test system. Therefore, CBRs affinity for both bivalent derivatives **6a-e** and monovalent ligands **7a-e** was measured by means of competition experiments by using the same radioligand (i.e. [³H]flumazenil), but different receptors and experimental binding setup. In particular, the binding of the compounds to the benzodiazepine site was measured in human recombinant $\alpha 1\beta 3\gamma 2$ and $\alpha 2\beta 3\gamma 2$ GABA_ARs. Thus, mouse L(tk) cells stably expressing human $\alpha 1\beta 3\gamma 2$ and $\alpha 2\beta 3\gamma 2$ GABA_ARs were generated by transfection of the individual subunits in the dexamethasone-inducible expression vector pMSGneo in mouse L(tk-) cells^{53,54} and were used in the binding assay by following a previously reported procedure (for the details see [Supporting Information](#)).

The results of the binding studies are summarized in [Table 2](#).

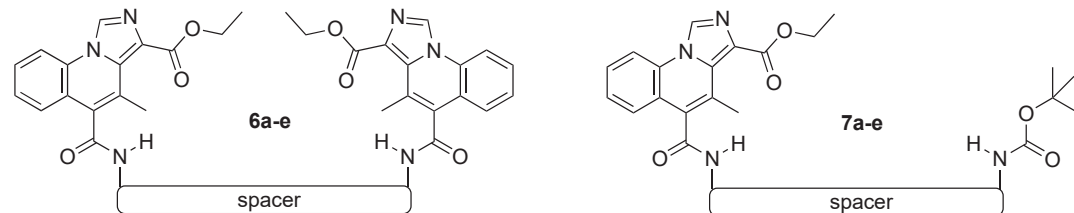
In this test system, the binding profiles of the tested compounds were rather different from the ones obtained in the experimental set-up employing cerebral membrane preparations from albino CD-1 mice. In fact, the tested compounds were found to inhibit the specific binding of radiolabeled flumazenil at the human recombinant $\alpha 1\beta 3\gamma 2$ and $\alpha 2\beta 3\gamma 2$ GABA_ARs showing roughly monophasic inhibition curves with *K_i* values from the sub-micromolar up to the nanomolar range very similar to that shown by diazepam (used as the reference compound in the binding assays). In particular, nanomolar *K_i* values were observed in both the series of bivalent **6a-c** and the corresponding monovalent ligands **7a-c**, with slightly better affinity values in the monovalent ligand series with respect to the bivalent series. Interestingly, the *K_i* values measured in this test system roughly correspond to those obtained for the low affinity site of CD-1 mice. Overall, these results appeared to rule out significant bivalent interactions and rather suggest monovalent interactions of these ligands with CBR. However, the role of each parameter of the experimental set-up (i.e. brain areas, incubation temperature and time, radioligand etc.) should be carefully investigated in a systematic pharmacological work to understand the molecular bases of the biphasic binding of these compounds in CD-1 mice.

2.2. In vivo efficacy

The selection of the compounds for the *in vivo* tests represented a very complex task because of the complexity of the binding profiles shown by the newly synthesized molecules. Overall, two bivalent ligands (i.e. **6a** and **6c**) and a reference monovalent ligand (i.e. **7a**) were selected as the most representatives of the most interesting compounds to be examined in mice for their pharmacological effects. Four potential benzodiazepine actions were considered: the anticonvulsant action evaluated by means of the compounds against pentylenetetrazole-induced convulsions, the potential anxiolytic effects screened using the light/dark box test, the myorelaxant effect with the rota rod test, and finally the mouse learning and memory impairment evaluated by the passive avoidance test. The anticonvulsant activity of the compounds was studied by means of pentylenetetrazole [6,7,8,9-tetrahydro-5H-tetrazoloazepine (PTZ)] as a chemical convulsant agent ([Table 3](#)).

Table 2

Inhibition of [³H]flumazenil specific binding to CBR in mouse L(tk-) cells stably expressing human recombinant $\alpha 1\beta 3\gamma 2$ and $\alpha 2\beta 3\gamma 2$ GABA_ARs of bivalent ligands **6a-e** and reference monovalent ligands **7a-e**.^a



Compd	Spacer	Methyleneequivalents	<i>K_i</i> (nM) $\alpha 1\beta 3\gamma 2$	<i>K_i</i> (nM) $\alpha 2\beta 3\gamma 2$
6a	-(CH ₂) ₆ -	6	74	45
6b	-(CH ₂) ₇ -	7	74	74
6c	-(CH ₂) ₈ -	8	64	56
6d	-(CH ₂ -CH ₂ -O) ₂ -CH ₂ -CH ₂ -	8	129	100
6e	-(CH ₂ -CH ₂ -O) ₇ -CH ₂ -CH ₂ -	23	245	210
7a	-(CH ₂) ₆ -	6	10	14
7b	-(CH ₂) ₇ -	7	31	33
7c	-(CH ₂) ₈ -	8	39	42
7d	-(CH ₂ -CH ₂ -O) ₂ -CH ₂ -CH ₂ -	8	615	400
7e	-(CH ₂ -CH ₂ -O) ₇ -CH ₂ -CH ₂ -	23	42	63
Diazepam			31	22

^a Cell membranes were incubated with 4 nM [³H]flumazenil ([³H]Ro 15-1788) in the absence and presence of increasing concentrations of the indicated compounds. All incubations were performed for 1 h at 4 °C in the assay buffer. Non-specific binding was determined in the presence of 1-3 μ M of **TP003** (see the Supporting Information for details). The % inhibition of [³H]Ro15-1788 binding was plotted as a function of compound concentration and the IC₅₀ calculated. From the IC₅₀ values, the *K_i* values were calculated using the method of Cheng and Prusoff using the *K_d* values obtained for [³H]Ro15-1788.

Table 3
Effect of **6a**, **6c**, and **7a** on convulsions induced by pentylenetetrazole.^a

Treatments	Dose mg/kg	Convulsion latency (min)	Death latency (min)
vehicle		19.3 ± 4.1	28.4 ± 4.8
6a	20	22.8 ± 5.5	40.5 ± 4.9*
6c	20	20.7 ± 4.8	41.8 ± 6.5*
7a	20	24.2 ± 3.9	43.3 ± 7.1*

^a Pentylenetetrazole (90 mg/kg i.p.) was injected 30 min before the administration of compounds. Each value represents the mean ± s.e.m. of at least 8 mice per group. *P<0.05 vs vehicle-treated animals.

As shown in Table 3, 20 mg/kg of **6a**, **6c**, and **7a** were able to significantly delay the mouse death latency in comparison to the control animals treated with vehicle. No effect was recorded concerning the time of the onset of convulsions (convulsion latency).

On the other hand, the effects of compounds on mouse anxiety in comparison to diazepam was evaluated using the light/dark box apparatus, the results are summarized in Table 4.

All compounds were challenged at the doses of 10 mg/kg and 20 mg/kg while diazepam was administered at 1 mg/kg. The time spent by vehicle-treated animals in the light box was 112.1 ± 10.9 s, this value was not changed by the treatment with **6a**, **6c**, and **7a**, at 10 mg/kg (114.3 ± 9.5 s, 96.7 ± 8.4 s, and 109.7 ± 8.7 s, respectively). All compounds at the higher dose potentiated this parameter up to 166.2 ± 8.2 s for **6a**, 173.4 ± 9.5 s for **6c**, and 144.7 ± 11.2 s for **7a**. Bivalent ligands **6a** and **6c**, and diazepam significantly increased the persistent time in light box up to 174.9 ± 10.9 s.

The effect of the acute administration of compounds (20 mg/kg, p.o.) on motor coordination was assessed using the rota rod test as a screening method to highlight any myorelaxant effect (Table 5).

Mice were challenged to perform the test before and after treatments (every 15 min) and the number of falls in 30 sec were counted. All compounds did not show a muscle relaxant effect since no increasing number of falls were detected after treatments in comparison to control animals injected with vehicle.

Finally, to investigate the effect of compounds on learning and memory, mice performance on passive avoidance test was analysed. This test allows to analyse if compounds show amnesic effects in a similar way to benzodiazepines. The difference between the retention latencies of compounds-treated mice and vehicles-treated mice was not statistically significant, meaning of no negative effect on learning and memory. On the contrary, the reference drug diazepam showed an amnesic effect after injection at the dose of 1 mg/kg (Table 6).

3. Conclusions

The imidazo[1,5-a]quinoline scaffold already reported in high affinity CBR ligands⁴³ was here employed as the pharmacophore in the design of bivalent CBR ligands **6a-e** bearing spacers showing variable length from six to twenty-three methylene equivalents and different

Table 4
Effect of **6a**, **6c**, and **7a** in comparison to diazepam in the light/dark box test.^a

Treatments	Dose mg/kg	Time in light (sec)
Vehicle		112.1 ± 10.9
6a	10	114.3 ± 9.5
6a	20	166.2 ± 8.2**
6c	10	96.7 ± 8.4
6c	20	173.4 ± 9.5**
7a	10	109.7 ± 8.7
7a	20	144.7 ± 11.2**
diazepam	1.0	174.9 ± 10.9**

^a All compounds were administered 30 min before the test. Each value represents the mean ± s.e.m. of at least 8 mice per group. **P<0.01 vs vehicle-treated animals.

Table 5
Lack of effect of **6a**, **6c**, and **7a** on mice rota rod test (falls in 30 sec).^a

Treatments	Dose mg/kg	Time (min)				
		0	15	30	45	60
Vehicle		5.0 ± 0.4	3.1 ± 0.4	1.6 ± 0.0	1.0 ± 0.2	0.2 ± 0.3
	6a	4.9 ± 0.3	3.2 ± 0.3	1.9 ± 0.4	0.7 ± 0.2	0.1 ± 0.2
6c		4.5 ± 0.4	3.0 ± 0.3	1.4 ± 0.2	0.7 ± 0.3	0.2 ± 0.3
	7a	5.1 ± 0.3	3.3 ± 0.3	1.8 ± 0.3	0.8 ± 0.2	0.2 ± 0.2

^a Each value represents the mean ± s.e.m. of at least 8 mice per group.

Table 6
Lack of effect of **6a**, **6c**, and **7a** in the mouse passive avoidance test.^a

Treatments	Dose mg/kg	Training session (s)	Retention session (s)	Δ
Saline		15.6 ± 3.2	92.4 ± 8.7	76.8
CMC		16.6 ± 3.5	90.7 ± 9.2	74.1
6a	20	15.3 ± 4.1	86.1 ± 7.1	70.8
6c	20	14.8 ± 3.2	91.8 ± 9.1	77.0
7a	20	18.1 ± 2.9	95.5 ± 9.3	77.4
Diazepam	1.0	17.4 ± 3.8	61.0 ± 6.8**	43.6

^a All compounds and diazepam were administered immediately after punishment. Each value represent the mean ± s.e.m. of at least 8 mice per group. **P<0.01 in comparison to saline/CMC treated mice.

physicochemical features. The newly designed compounds **6a-e** were synthesized exploiting previously developed procedures along with the corresponding reference monovalent compounds **7a-e** bearing the corresponding spacers terminated with a *tert*-butoxycarbonyl (Boc) group. The affinity to GABA_ARs was evaluated in binding assays by using different CBR preparations (such as the cerebral cortex from male CD-1 albino mice or the human recombinant α1β3γ2 and α2β3γ2 receptors stably expressed in mouse L(tk-) cells) and different experimental set-up. The results obtained in the human recombinant α1β3γ2 and α2β3γ2 GABA_ARs showed that the tested compounds were able to inhibit radiolabelled flumazenil, displaying monophasic inhibition curves, with *K_i* values from the sub-micromolar up to the nanomolar range. The similar inhibition constants values at α1- and α2-subunit containing GABA_ARs showed an apparent lack of selectivity across the two isoforms. Nano-molar *K_i* values were obtained in both the series of homo-bivalent **6a-c** and the corresponding monovalent ligands **7a-c**, which showed slightly better affinity values. The similarity in the affinity between the bivalent ligands and the corresponding monovalent ones appeared to rule out any bivalent interactions of these ligands with the two isoforms of GABA_ARs. Similarly, both the series of bivalent **6a-c** and the corresponding monovalent ligands **7a-c** were able to inhibit the radiolabeled flumazenil to GABA_ARs in cortical membranes of albino CD-1 mice, but most of the tested compounds showed biphasic inhibition curves, which suggested the existence of two well-distinct populations of binding sites. Interestingly, the inhibition constant values measured for the low affinity site roughly correspond to those obtained in the human recombinant α1β3γ2 and α2β3γ2 GABA_ARs. Surprisingly, the apparent affinity of some of the tested compound for the high affinity site was estimated to be in the sub-picomolar range. Giving the lack of an unconfutable explanation for this surprising results, further studies are obviously necessary to evaluate the reproducibility of this observation. Finally, some CBR ligands selected from the bivalent ligands (i.e. **6a,c**) and from the reference monovalent ligands (i.e. **7a**) were then tested for their potential pharmacological *in vivo* effects in mice. Four potential benzodiazepine actions such as anticonvulsant, anxiolytic, locomotor, and anti-amnesic activities were taken into consideration. All the tested compounds showed anticonvulsant and anxiolytic properties without muscle relaxant and learning and memory negative effects.

CRedit authorship contribution statement

Marco Paolino: Writing – original draft, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Mario Saletti:** Formal analysis. **Jacopo Venditti:** Formal analysis. **Federica Castriconi:** Investigation, Formal analysis. **Germano Giuliani:** Investigation, Formal analysis. **Samuele Maramai:** Writing – review & editing, Formal analysis, Data curation. **Alessandra Toti:** Investigation, Formal analysis, Data curation. **Carla Ghelardini:** Methodology, Investigation, Formal analysis, Data curation. **Rosanna Matucci:** Methodology, Investigation, Formal analysis, Data curation. **Narcy Alcazar Villalobos:** Methodology, Investigation, Formal analysis, Data curation. **Maurizio Anzini:** Project administration, Investigation. **Andrea Cappelli:** Writing – original draft, Supervision, Project administration, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Chemical procedures and characterization data of compounds **6a-e** and **7a-e**. Experimental details for *in vitro* and *in vivo* studies. This material is available free of charge via the Internet at. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmc.2024.118006>.

Data availability

Data will be made available on request.

References

- Thompson AJ, Lester HA, Lummis SCR. The structural basis of function in Cys-loop receptors. *Q Rev Biophys.* 2010;43:449–499. <https://doi.org/10.1017/S0033583510000168>.
- Smith GB, Olsen RW. Functional domains of GABA_A receptors. *Trends Pharmacol Sci.* 1995;16:162–168. [https://doi.org/10.1016/S0165-6147\(00\)89009-4](https://doi.org/10.1016/S0165-6147(00)89009-4).
- Argyropoulos SV, Nutt DJ. The use of benzodiazepines in anxiety and other disorders. *Eur Neuropsychopharmacol.* 1999;9:S407–S412. [https://doi.org/10.1016/S0924-977X\(99\)00052-8](https://doi.org/10.1016/S0924-977X(99)00052-8).
- Park-Chung M, Malayev A, Purdy RH, Gibbs TT, Farb DH. Sulfated and unsulfated steroids modulate γ -aminobutyric acid A receptor function through distinct sites. *Brain Res.* 1999;830:72–87. [https://doi.org/10.1016/S0006-8993\(99\)01381-5](https://doi.org/10.1016/S0006-8993(99)01381-5).
- Rupprecht R, Holsboer F. Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci.* 1999;22:410–416. [https://doi.org/10.1016/S0166-2236\(99\)01399-5](https://doi.org/10.1016/S0166-2236(99)01399-5).
- Dorow R. FG 7142 and its anxiety-inducing effects in humans. *Br J Clin Pharmacol.* 1987;23:781–782.
- Williams TJ, Bowie PE. Midazolam sedation to produce complete amnesia for bronchoscopy: 2 years' experience at a district general hospital. *Respir Med.* 1999;93:361–365. [https://doi.org/10.1016/S0954-6111\(99\)90320-7](https://doi.org/10.1016/S0954-6111(99)90320-7).
- Barker MJ, Jackson M, Greenwood KM, Crowe SF. Cognitive effects of benzodiazepine use: a review. *Aust Psychol.* 2003;38:202–213. <https://doi.org/10.1080/00050060310001707217>.
- Lister RG. The amnesic action of benzodiazepines in man. *Neurosci Biobehav Rev.* 1985;9:87–94. [https://doi.org/10.1016/0149-7634\(85\)90034-X](https://doi.org/10.1016/0149-7634(85)90034-X).
- Curran HV. Tranquillising memories: a review of the effects of benzodiazepines on human memory. *Biol Psychol.* 1986;23:179–213. [https://doi.org/10.1016/0301-0511\(86\)90081-5](https://doi.org/10.1016/0301-0511(86)90081-5).
- Chambers MS, Atack JR, Broughton HB, et al. Identification of a novel, selective GABA_A α 5 receptor inverse agonist which enhances cognition. *J Med Chem.* 2003;46:2227–2240. <https://doi.org/10.1021/jm020582q>.
- Sarter M, Bruno JP, Berntson GG. Psychotogenic properties of benzodiazepine receptor inverse agonists. *Psychopharmacology.* 2001;156:1–13. <https://doi.org/10.1007/s002130100756>.
- Dorow R, Horowski R, Paschelke G, Amin M, Braestrup C. Severe anxiety induced by FG 7142, A β -carboline ligand for benzodiazepine receptors. *Lancet.* 1983;322:98–99. [https://doi.org/10.1016/S0140-6736\(83\)90076-4](https://doi.org/10.1016/S0140-6736(83)90076-4).
- Barnard EA, Skolnick P, Olsen RW, et al. International Union of Pharmacology. XV. Subtypes of γ -Aminobutyric Acid <sub>A</sub> receptors: classification

- on the basis of subunit structure and receptor function. *Pharmacol Rev* 1998;50(2): 291 LP - 3http://pharmrev.aspetjournals.org/content/50/2/291.abstract.
- Knoflach F, Rhyner T, Villa M, et al. The γ 3-subunit of the GABA_A-receptor confers sensitivity to benzodiazepine receptor ligands. *FEBS Lett.* 1991;293:191–194. [https://doi.org/10.1016/0014-5793\(91\)81184-A](https://doi.org/10.1016/0014-5793(91)81184-A).
 - Ernst M, Brauchart D, Boresch S, Sieghart W. Comparative modeling of GABA_A receptors: limits, insights, future developments. *Neuroscience.* 2003;119:933–943. [https://doi.org/10.1016/S0306-4522\(03\)00288-4](https://doi.org/10.1016/S0306-4522(03)00288-4).
 - McKernan RM, Rosahl TW, Reynolds DS, et al. Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA_A receptor α 1 subtype. *Nat Neurosci.* 2000;3:587–592. <https://doi.org/10.1038/75761>.
 - Rudolph U, Möhler H. Analysis of GABA_A receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annu Rev Pharmacol Toxicol.* 2004;44:475–498. <https://doi.org/10.1146/annurev.pharmtox.44.101802.121429>.
 - Atack JR, Hutson PH, Collinson N, et al. Anxiogenic properties of an inverse agonist selective for α 3 subunit-containing GABA_A receptors. *Br J Pharmacol.* 2005;144: 357–366. <https://doi.org/10.1038/sj.bjp.0706056>.
 - Rudolph U, Knoflach F. Beyond classical benzodiazepines: novel therapeutic potential of GABA_A receptor subtypes. *Nat Rev Drug Discov.* 2011;10:685–697. <https://doi.org/10.1038/nrd3502>.
 - Gill KM, Lodge DJ, Cook JM, Aras S, Grace AA. A novel α 5GABAAR-positive allosteric modulator reverses hyperactivation of the dopamine system in the MAM model of schizophrenia. *Neuropsychopharmacology.* 2011;36:1903–1911. <https://doi.org/10.1038/npp.2011.76>.
 - Cook JM, Clayton TS, Jain HD, et al. Gabaergic receptor subtype selective ligands and their uses; 2015:US 20150258128 A1.
 - Cook JM, Clayton TS, Jain HD, et al. Gabaergic receptor subtype selective ligands and their uses; 2012:US 20120295892 A1.
 - He X, Huang Q, Ma C, Yu S, McKernan R, Cook JM. Pharmacophore/receptor models for GABA(A)/BzR α 2 β 3 γ 2, α 3 β 3 γ 2 and α 4 β 3 γ 2 recombinant subtypes. Included volume analysis and comparison to α 1 β 3 γ 2, α 5 β 3 γ 2, and α 6 β 3 γ 2 subtypes. *Drug Des Discov.* 2000;17:131–171.
 - Clayton T, Poe MM, Rallapalli S, et al. A Review of the updated pharmacophore for the α 5 GABA(A) benzodiazepine receptor model. *Int J Med Chem.* 2015;2015, 430248. <https://doi.org/10.1155/2015/430248>.
 - Savić MM, Milinković MM, Rallapalli S, et al. The differential role of α 1- and α 5-containing GABA_A receptors in mediating diazepam effects on spontaneous locomotor activity and water-maze learning and memory in rats. *Int J Neuropsychopharmacol.* 2009;12:1179–1193. <https://doi.org/10.1017/S1461145709000108>.
 - Drexler B, Zinser S, Huang S, et al. Enhancing the function of α 5-subunit-containing GABA_A receptors promotes action potential firing of neocortical neurons during up-states. *Eur J Pharmacol.* 2013;703:18–24. <https://doi.org/10.1016/j.ejphar.2013.01.034>.
 - Maramai S, Benchekroun M, Ward SE, Atack JR. Subtype selective γ -aminobutyric acid type A receptor (GABAAR) modulators acting at the benzodiazepine binding site: an update. *J Med Chem.* 2020;63:3425–3446. <https://doi.org/10.1021/acs.jmedchem.9b01312>.
 - Anzini M, Braile C, Valenti S, et al. Ethyl 8-fluoro-6-(3-nitrophenyl)-4h-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate as novel, highly potent, and safe antianxiety agent. *J Med Chem.* 2008;51:4730–4743. <https://doi.org/10.1021/jm8002944>.
 - Anzini M, Valenti S, Braile C, et al. New insight into the central benzodiazepine receptor–ligand interactions: design, synthesis, biological evaluation, and molecular modeling of 3-substituted 6-phenyl-4H-imidazo[1,5-a][1,4]benzodiazepines and related compounds. *J Med Chem.* 2011;54:5694–5711. <https://doi.org/10.1021/jm2001597>.
 - Petke JD, Im HK, Im WB, et al. Characterization of functional interactions of imidazoquinoline derivatives with benzodiazepine-gamma-aminobutyric acidA receptors. *Mol Pharmacol.* 1992;42(2):294 LP - 301. <http://molpharm.aspetjournals.org/content/42/2/294.abstract>.
 - TenBrink RE, Im WB, Sethy VH, Tang AH, Carter DB. Antagonist, partial agonist, and full agonist imidazo[1,5-a]quinoxaline amides and carbamates acting through the GABA_A/benzodiazepine receptor. *J Med Chem.* 1994;37:758–768. <https://doi.org/10.1021/jm00032a008>.
 - Jacobsen EJ, TenBrink RE, Stelzer LS, et al. High-affinity partial agonist imidazo[1,5-a]quinoxaline amides, carbamates, and ureas at the γ -aminobutyric acid A/benzodiazepine receptor complex. *J Med Chem.* 1996;39:158–175. <https://doi.org/10.1021/jm940765f>.
 - Jacobsen EJ, Stelzer LS, Belonga KL, et al. 3-Phenyl-substituted imidazo[1,5-a]quinoxalin-4-ones and imidazo[1,5-a]quinoxaline ureas that have high affinity at the GABA_A/benzodiazepine receptor complex. *J Med Chem.* 1996;39:3820–3836. <https://doi.org/10.1021/jm960070-1>.
 - Mickelson JW, Jacobsen EJ, Carter DB, et al. High-affinity α -aminobutyric acid A/benzodiazepine ligands: synthesis and structure–activity relationship studies of a new series of tetracyclic imidazoquinoxalines. *J Med Chem.* 1996;39:4654–4666. <https://doi.org/10.1021/jm960401i>.
 - Jacobsen EJ, Stelzer LS, TenBrink RE, et al. Piperazine imidazo[1,5-a]quinoxaline ureas as high-affinity GABA_A ligands of dual functionality. *J Med Chem.* 1999;42: 1123–1144. <https://doi.org/10.1021/jm9801307>.
 - Im HK, Bin Im W, Pregenzer JF, Stratman NC, VonVoigtlander PF, Jon JE. Two imidazoquinoline ligands for the benzodiazepine site sharing a second low affinity site on rat GABA_A receptors but with the opposite functionality. *Br J Pharmacol.* 1998;123:1490–1494. <https://doi.org/10.1038/sj.bjp.0701763>.

38. Zhong WZ, Williams MG, Branstetter DG. Toxicokinetics in drug development: an overview of toxicokinetic application in the development of PNU-101017, an anxiolytic drug candidate. *Curr Drug Metab.* 2000;1:243–254.
39. Cappelli A, Gallelli A, Manini M, et al. Further studies on the interaction of the 5-hydroxytryptamine₃ (5-HT₃) receptor with arylpiperazine ligands. development of a new 5-HT₃ receptor ligand showing potent acetylcholinesterase inhibitory properties. *J Med Chem.* 2005;48:3564–3575. <https://doi.org/10.1021/jm0493461>.
40. Cappelli A, Butini S, Brizzi A, et al. The interactions of the 5-HT₃ receptor with quipazine-like arylpiperazine ligands. The journey track at the end of the first decade of the third millennium. *Curr Top Med Chem.* 2010;10:504–526.
41. Cappelli A, Manini M, Paolino M, et al. Bivalent ligands for the serotonin 5-HT₃ receptor. *ACS Med Chem Lett.* 2011;2:571–576. <https://doi.org/10.1021/ml2000388>.
42. Paolino M, Mennuni L, Giuliani G, et al. Dendrimeric tetravalent ligands for the serotonin-gated ion channel. *Chem Commun (Camb).* 2014;50:8582–8585. <https://doi.org/10.1039/c4cc02502d>.
43. Cappelli A, Anzini M, Castriconi F, et al. Design, synthesis, and biological evaluation of imidazo[1,5-a]quinoline as highly potent ligands of central benzodiazepine receptors. *J Med Chem.* 2016;59:3353–3372. <https://doi.org/10.1021/acs.jmedchem.6b00034>.
44. Galeazzi S, Hermans TM, Paolino M, et al. Multivalent supramolecular dendrimer-based drugs. *Biomacromolecules.* 2010;11:182–186.
45. Paolino M, Komber H, Mennuni L, et al. Supramolecular glycodendrimer-based hybrid drugs. *Biomacromolecules.* 2014;15:3985–3993. <https://doi.org/10.1021/bm501057d>.
46. Castriconi F, Paolino M, Donati A, et al. Multivalent ligands for the serotonin 5-HT₄ receptor. *Med Chem Commun.* 2017;8:647–651. <https://doi.org/10.1039/C6MD00458J>.
47. Pringle AK, Gardner CR, Walker RJ. Biphasic actions of the benzodiazepine receptor inverse agonist ru34347 in the rat cerebellar slice. *Brain Res.* 1996;715:155–162. [https://doi.org/10.1016/0006-8993\(95\)01571-X](https://doi.org/10.1016/0006-8993(95)01571-X).
48. McLeod M, Pralong D, Copolov D, Dean B. The heterogeneity of central benzodiazepine receptor subtypes in the human hippocampal formation, frontal cortex and cerebellum using [³H]flumazenil and zolpidem. *Mol Brain Res.* 2002;104:203–209. [https://doi.org/10.1016/S0169-328X\(02\)00381-9](https://doi.org/10.1016/S0169-328X(02)00381-9).
49. Byrnes JJ, Greenblatt DJ, Miller LG. Benzodiazepine receptor binding of nonbenzodiazepines in vivo: alpidem, zolpidem and zopiclone. *Brain Res Bull.* 1992;29:905–908. [https://doi.org/10.1016/0361-9230\(92\)90164-S](https://doi.org/10.1016/0361-9230(92)90164-S).
50. Schmid L, Bottlaender M, Fuseau C, Fournier D, Brouillet E, Mazière M. Zolpidem displays heterogeneity in its binding to the nonhuman primate benzodiazepine receptor in vivo. *J Neurochem.* 1995;65:1880–1886. <https://doi.org/10.1046/j.1471-4159.1995.65041880.x>.
51. Brown CL, Martin IL. Kinetics of [³H]Ro 15–1788 binding to membrane-bound rat brain benzodiazepine receptors. *J Neurochem.* 1984;42:918–923. <https://doi.org/10.1111/j.1471-4159.1984.tb12691.x>.
52. Mehta AK, Shank RP. Characterization of a benzodiazepine receptor site with exceptionally high affinity for Ro 15-4513 in the rat CNS. *Brain Res.* 1995;704:289–297. [https://doi.org/10.1016/0006-8993\(95\)01133-1](https://doi.org/10.1016/0006-8993(95)01133-1).
53. Hadingham KL, Wingrove P, Le Bourdelles B, Palmer KJ, Ragan CI, Whiting PJ. Cloning of cDNA sequences encoding human alpha 2 and alpha 3 gamma-aminobutyric acidA receptor subunits and characterization of the benzodiazepine pharmacology of recombinant alpha 1-, alpha 2-, alpha 3-, and alpha 5-containing human gamma-aminobutyric ac. *Mol Pharmacol.* 1993;43(6):970 LP - 975. <http://molpharm.aspetjournals.org/content/43/6/970.abstract>.
54. Hadingham KL, Wingrove PB, Wafford KA, et al. Role of the beta subunit in determining the pharmacology of human gamma-aminobutyric acid type A receptors. *Mol Pharmacol.* 1993;44(6):1211 LP - 1218. <http://molpharm.aspetjournals.org/content/44/6/1211.abstract>.