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Development of ligands at γ-aminobutyrric acid type A ($GABA_A$) receptor subtype as new agents for pain relief

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ABSTRACT

The identification of compounds with selective anxiolytic-like effects, exerted through the benzodiazepine site on γ-aminobutyric acid type A ($GABA_A$) receptors, and that show pronounced antihyperalgesia in several pain models, has oriented research towards the development of new agents for the relief of pain. Starting from our previously reported ligands at the benzodiazepine site on $GABA_A$ receptors showing selective anxiolytic-like effects, we have designed new compounds with the aim of identifying those devoid of the typical side effects of the classical benzodiazepines. Our preliminary results indicate that compounds **4**, **10**(±) and **11** have a very promising antihyperalgesic profile in different animal pain models (peripheral mono-neuropathy, STZ-induced hyperalgesia). In particular **11** exhibits high potency since it exerted its protective effect starting from the dose of 3 mg/kg po, after single injection.

1. Introduction

The importance of the γ-aminobutyrric acid (GABA) neurotransmitter in mediating or modulating of many functions of the central nervous system cannot be questioned. It is involved in many physiological functions related to its key role in the fast synaptic inhibition in the brain and spinal cord via activation of synaptic or extrasynaptic ionotropic GABA receptors ($GABA_A$-receptors). Deficits in the functional expression of these receptors are critical in a large number of human neurological and psychiatric diseases such as epilepsy, anxiety disorder, cognitive deficits, schizophrenia, depression and pain.[1]

$GABA_A$-R is a pentameric arrangement of different subunits (α, β, γ, δ, ε, θ, π and ρ), many of which exist in multiple isoforms (α 1–6, β 1–3, γ 1–3, ρ 1–3), with a typical composition of 2α, 2β and 1γ or 1δ Activation of $GABA_A$-R causes an influx of chloride ion and hyperpolarization of the neuron. The allosteric modulation of $GABA_A$-Rs by benzodiazepine and/or non-benzodiazepine ligands induces conformational changes in the multi-subunit receptor that open the central channel and the chloride ions move into the cells. Especially for the ligands at the benzodiazepine site on $GABA_A$-Rs (otherwise called ligands to $GABA_A$-Rs subtype), situated at the interface between the alpha and gamma subunits, the conformational changes can produce either the potentiation (agonist or PAM, positive allosteric modulator) or the decrease (inverse agonist or NAM, negative allosteric modulator) in the response elicited by GABA or no event (antagonist).[2–4]

It is known that $GABA_A$-Rs are extensively distributed within peripheral and spinal nociceptive system[5] and play an important role in the mediation of pain transmission. Although a great variety of mediators are involved in pain pathologies, the loss of synaptic inhibition of glycinergic and/or GABAergic input after nerve injury or tissue trauma or inflammation (becoming less inhibitory or even excitatory) has been recognized as an important process in the development and maintenance of pain of various origin.[6] Even if the precise basis of this dis-inhibition may be attributed to different causes (loss of GABAergic interneurons, reduced GABA storage/release, alteration of cation-chloride co-transporter),[7,8] it is clear that restoring spinal synaptic inhibition, through pharmacological facilitation of $GABA_A$-Rs, is an interesting and intriguing approach for the treatment of pain.[9,10] Many authors have reported that benzodiazepines (mainly midazolam) modulate pain perception both in animal experiments[11–13] and in human patients[14,15] even though the complexity of the intrathecal administration precludes routine use. On the other hand, even though systemic administration of

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midazolam can induce antihyperalgesic action in the formalin test,[16] this effect can be masked or complicated by the sedative effects, thus reducing its use against pain.

Benzodiazepine-sensitive $GABA_A$-Rs subtypes contain four types of alpha isoform (α1–3 and α5), to which it has been possible to attribute the effects on sedation, anxiety and memory of diazepam (full agonist), using $GABA_A$-Rs point-mutated knock-in mice.[17–23] Using this type of approach it has recently been reported that the $GABA_A$-Rs containing α2 (in addition to the anxiolytic effect) are the principal contributors (and to a lesser extent -α3) to the antihyperalgesic actions of classical benzodiazepines (e.g., diazepam) administered intrathecally[24] or systemically.[25] This effect is evidenced when the pain sensitivity is increased pathologically (inflammation or neuropathic injury) in agreement with the notion that the benzodiazepines are generally not analgesic per se. Thus, the use of α2 selective agonist could be a useful tool for treatment of pain disorders as reported in several rodent pain models, in which pronounced antihyperalgesia was obtained,[25–28] without sedative effects.

Recently we have reported that some derivatives with the pyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide core have selective anxiolytic-like properties[29–32] without exhibiting sedative and myorelaxant effects. Since the relationship between anxiolysis and antihyperalgesia is intriguing we decided to investigate the antihyperalgesic profile of some compounds we have already reported as selective anxiolytics (**I**,[29] **II**,[32] and **III**,[30] see Chart 1).

Based on the pharmacological results of these compounds (data reported in the Supplementary data) the derivative with the most promising antihyperalgesic effects was **III**,[30] thus we synthesized new 3-iodio- and 3-methyl-, 8-aryalkyloxy- and/or 8-arylalkyl aminopyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide derivatives, as its optimization. Moreover, to better find out the features for antihyperalgesic activity, we decided to test two already reported[33,30] 3-iododerivatives in vivo, in this work compounds **9** and **10**(±). Clearly, to assess the involvement of $GABA_A$-Rs, the binding affinity and the potential anxiolytic-like effects were compared with those of diazepam and antagonized by flumazenil ($GABA_A$-R antagonist). Compounds having anxiolytic-like activity were then tested in adult rats on a pain model in which peripheral mono-neuropathy was produced; in the animal model of streptozotocin (STZ)-induced hyperalgesia, which mimics the pain caused by diabetic neuropathy, and in the mouse abdominal constriction test following injection of acetic acid 0.6%.

2. Chemistry

The 3-methyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, **3** and the previous synthesized 3-iodo-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide[34] were used as starting material for the synthesis of the corresponding 3-methyl-(compounds **4–8**) and 3-iodo-(compounds **11–15**), either 8-arylalkyloxy- or 8-arylalkylamino derivatives.

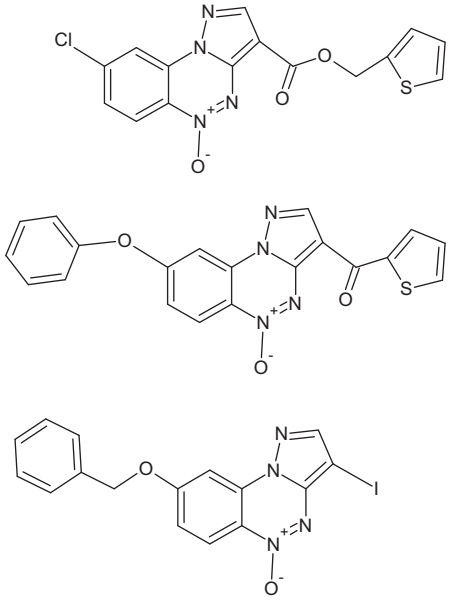
Compound **3**, Scheme 1, was obtained by exploiting the condensation between the 2-methyl-3-oxo-propanenitrile sodium salt (sodium 2-cyanoprop-1-en-1-olate) and 2-nitro-5-chlorophenylhydrazine in ethanol solution at pH 1 (with hydrochloric acid). During the reaction was evidenced the formation of two products that, identified step by step, resulted to be the 1-(2-nitro-5-chlorophenyl)-2*H*-4-methyl-5-iminopyrazole **1** and the 1-(2-nitro-5-chlorophenyl)-4-methyl-5-aminopyrazole **2**. Both the products cyclized to 3-methyl-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide, **3** when reacted in 10% sodium hydroxide solution (see Scheme 1).

The 8-arylalkyloxy derivatives **4**, **5**, **6**(±) were obtained by exploiting the nucleophilic substitution of the chlorine atom at position 8 of the starting materials **3**, by using the suitable alcohols, with phase transfer catalyst (PTC). The 8-arylalkylamino derivatives, **7–8**, **11–15**, were instead obtained by treating the starting materials **3** and the 3-iodo-8-chloropyrazolo[5,1-*c*][1,2,4]benzotriazine 5-oxide,[34] with an excess of suitable amine and easily recovered as the principal compound at the end of the reaction, Scheme 2.

Compounds **4**, **7** and **11** were deoxidized at position 5 by treatment with zinc in acetic acid, obtaining respectively derivatives **4R**, **7R** and **11R**, useful for study of SAR, Scheme 3. All compounds described here are listed in Table 1.

3. Results and discussion

The Bz site/$GABA_A$-R binding affinity of newly synthesized compounds was evaluated by their ability to displace [^{3}H]flumazenil (Ro15–1788) from its specific binding in bovine brain membrane

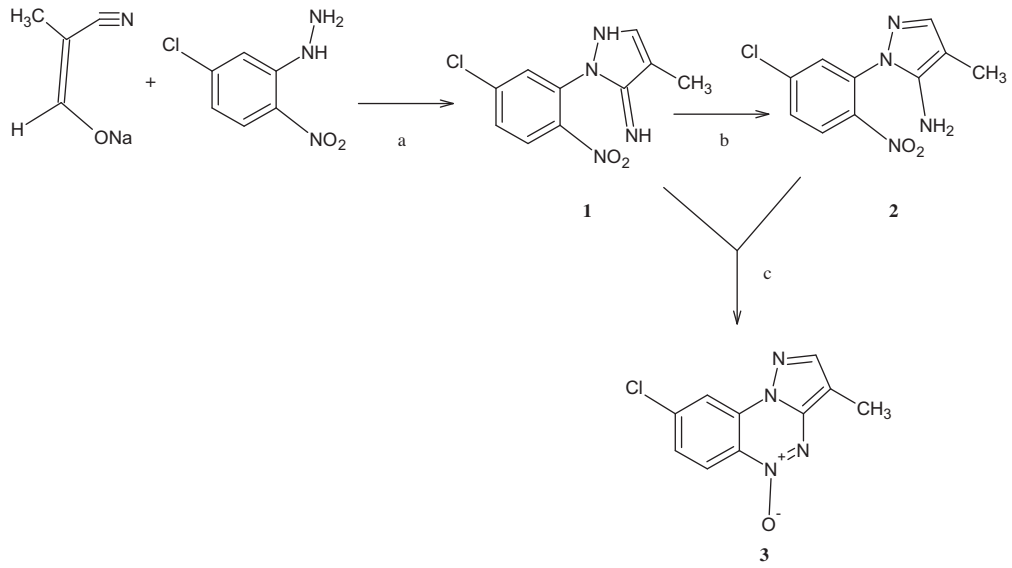


I[a]
K_i = 6.8 nM
Anxiolytic-like effect at 1-100 mg/kg

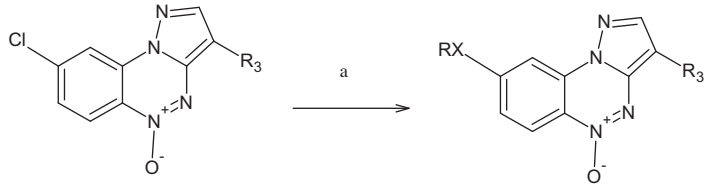
II[b]
K_i = 8.9 nM
Anxiolytic-like effect at 10-30 mg/kg

III[c]
K_i = 8.9 nM
Anxiolytic-like effect at 3-30 mg/kg

Chart 1. see Ref.[29]; [b]see Ref.[32]; [c]see Ref.[30]



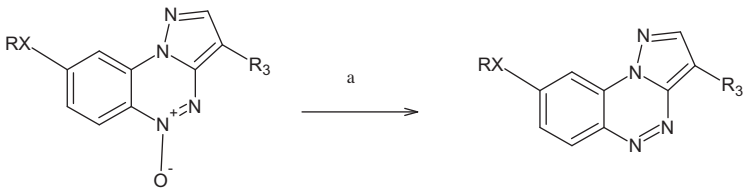
Scheme 1. Reagents and conditions: (a) Ethanol /HCl, 80 °C 2 h; (b) Ethanol /HCl, 80 °C 10 h; (c) NaOH 10%, 50 °C.



$R_3 = CH_3$	3
$R_3 = I$	see ref.[34]

Comp	X	R	R_3
4	O	CH_2Ph	CH_3
5	O	CH_2-*o*-OCH_3Ph	CH_3
6(±)	O	$CH_2(CH)CH_3Ph$	CH_3
7	NH	CH_2Ph	CH_3
8	NH	CH_2-*o*-OCH_3Ph	CH_3
11	NH	CH_2Ph	I
12	NH	CH_2-*o*-OCH_3Ph	I
13	NH	CH_2-*p*-ClPh	I
14	NH	CH_2CH_2Ph	I
15	NCH_3	CH_2Ph	I

Scheme 2. Reagents: (a) ROH/NaOH 40% solution/NBu^+Br^-/CH_2Cl_2 for compounds **4**–**6(±)**; RNH_2/EtOH for compounds **7**–**8**, **11**–**15**.

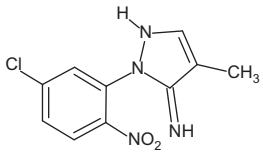


Comp	X	R	R_3
4	O	CH_2Ph	CH_3
7	NH	CH_2Ph	CH_3
11	NH	CH_2Ph	I

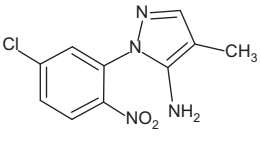
Comp	X	R	R_3
4R	O	CH_2Ph	CH_3
7R	NH	CH_2Ph	CH_3
11R	NH	CH_2Ph	I

Scheme 3. Reagents: (a) Acetic acid/zinc dust

Table 1
Chemical data for new synthesized compounds



1



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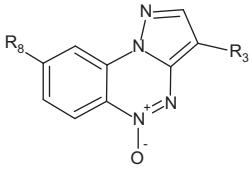
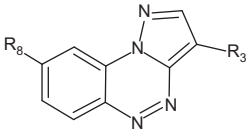


3-15



4R, 7R, 11R

Compd	R^3	R^8	Formula (MW)	Mp °C (recryst. solvent)
1	—	—	$C_{10}H_9N_4O_2Cl$ (252.66)	176–179
2	—	—	$C_{10}H_9N_4O_2Cl$ (252.66)	247–248 (Ethanol)
3	CH_3	Cl	$C_{10}H_7N_4OCl$ (234.64)	246–247 (Ethanol)
4	CH_3	OCH_2-Ph	$C_{17}H_{14}N_4O_2$ (306.32)	207–208 (Ethanol)
4R[a]	CH_3	OCH_2-Ph	$C_{17}H_{14}N_4O$ (290.31)	236–237 (Ethanol)
5	CH_3	OCH_2-*o*-OCH_3-Ph	$C_{18}H_{16}N_4O_3$ (336.34)	247–249 (Methoxyethanol)
6(±)	CH_3	$OCH_2CH(CH_3)$-Ph	$C_{19}H_{18}N_4O_2$ (334.38)	133–134 (Ethanol)
7	CH_3	$NHCH_2$-Ph	$C_{17}H_{15}N_5O$ (305.33)	>300 (Chromathography)
7R[a]	CH_3	$NHCH_2$-Ph	$C_{17}H_{15}N_5$ (289.33)	236–237 (Ethanol)
8	CH_3	$NHCH_2$-*o*-OCH_3-Ph	$C_{18}H_{17}N_5O_2$ (335.36)	254–255 (Chromathography)
9[b]	I	$O(CH_2)_4CH_3$		
10(±)[c]	I	$OCH_2CH(CH_3)$-Ph		
11	I	$NHCH_2$-Ph	$C_{16}H_{12}N_5OI$ (417.20)	239–240 (Methoxyethanol)
11R[a]	I	$NHCH_2$-Ph	$C_{16}H_{12}N_5I$ (401.20)	242–243 (Methoxyethanol)
12	I	$NHCH_2$-*o*-OCH_3-Ph	$C_{17}H_{14}N_5O_2I$ (447.23)	238–239 (Ethanol)
13	I	$NHCH_2$-*p*-Cl-Ph	$C_{16}H_{11}N_5OICl$ (451.65)	>300 (Methoxyethanol)
14	I	$NHCH_2CH_2$-Ph	$C_{17}H_{14}N_5OI$ (431.23)	240–241 (Ethanol)
15	I	$N(CH_3)$ CH_2-Ph	$C_{17}H_{14}N_5OI$ (431.23)	221–223 (Methoxyethanol)

[a] 5-Deoxide derivatives.
[b] See Ref. 33.
[c] See Ref. 30.

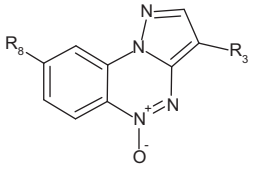
and was expressed as K_i value only for those compounds inhibiting radioligand binding by more than 80% at fixed concentrations of 10 μM. The binding data (Table 2) show that all compounds were able to bind to the Bz site/$GABA_A$-R with good affinity, reaching, for some derivatives **8**, **11**, **11R** and **12**, the subnanomolar range ($0.78 \geqslant K_i$ (nM) $\geqslant 0.109$).

Compounds **3**, **4**, **5**, **6(±)**, endowed with a 3-methyl group on the pyrazolobenzotriazine 5-oxide system, show affinity in the range of 400 K_i (nM) $\geqslant 3.02$. The importance of the oxygen atom at position 8[30,33] is evidenced by the weak binding affinity of compound **3** (K_i = 400 nM), the 8-chloro derivative, lacking of 8-oxygen atom. Moreover also the derivative 8-(2-methyl-2-phenylethoxy)-, **6(±)**, probably because of a steric hindrance of the branched 8-*O*-benzyl chain (K_i = 361.2 nM), has weak affinity. These compounds have lower affinity than the corresponding 3-iodo derivatives (8-chloro, K_i = 22.8 nM and 8-(2-methyl-2-phenylethoxy)- K_i = 160 nM respectively,[30]). This fact is probably due to reduced ability of the methyl group to form a lipophilic interaction with the receptor protein, as also indicated by the different value of the lipophilic parameter, π: methyl, $\pi = 0.56$ and iodine, $\pi = 1.12$,.[35] The 3-methyl derivatives (**4** and **5**) show good affinity values (**4**, K_i = 4.66 nM and **5**, K_i = 3.02 nM) even if reduced by about 4–7-fold compared to the corresponding 3-iodo derivatives (8-benzyloxy- K_i = 1.1 nM and 8-*o*-methoxybenzyloxy- K_i = 0.42 nM[30]), confirming the importance of more lipophilic substituent at position 3.

When the position 8 was substituted with a moiety that contains a hydrogen bond donor atom (–NH–), in the 3-methyl derivatives (**7–8**) the affinity values improved 2–4-fold compared to the corresponding 8-*O*-derivatives (**4–5**). Compare compound **7**, K_i = 2.58 nM with **4**, K_i = 4.66 nM and compound **8**, K_i = 0.78 nM with **5**, K_i = 3.02 nM, suggesting a positive influence of the NH group.

On the other hand, when the position 3 of the 8-aryl-alkylaminopyrazolobenzotriazine core was again substituted with a more lipophilic substituent, that is, iodine, compounds **11**, **12** and **13** emerge with very good affinity value: **11**, K_i = 0.109 nM; **12**, K_i = 0.138 nM; **13**, K_i = 2.44 nM. These results confirm the importance of the lipophilic feature of the substituent at position 3 and, again, the key role of the hydrogen bond donor moiety (8-NH- group) to better receptor recognition. The modification of

Table 2
Binding data of new compounds



Compd	R^3	R^8	K_i[a] (nM)
3	CH_3	Cl	400 ± 11.0
4	CH_3	OCH2-Ph	4.66 ± 0.02
4R[b]	CH_3	OCH2-Ph	1.60 ± 0.07
5	CH_3	OCH2-o-OCH3-Ph	3.02 ± 0.2
6(±)	CH_3	OCH2CH(CH3)-Ph	361.2 ± 25.6
7	CH_3	NHCH2-Ph	2.58 ± 0.13
7R[b]	CH_3	NHCH2-Ph	2.78 ± 0.06
8	CH_3	NHCH2-o-OCH3-Ph	**0.78 ± 0.04**
9[c]	I	O(CH2)4CH3	6.5 ± 0.4
10(±)[d]	I	OCH2CH(CH3)-Ph	160 ± 15.0
11	I	NHCH2-Ph	**0.109 ± 0.008**
11R[b]	I	NHCH2-Ph	**0.71 ± 0.07**
12	I	NHCH2-o-OCH3-Ph	**0.138 ± 0.005**
13	I	NHCH2-p-Cl-Ph	2.44 ± 0.1
14	I	NHCH2CH2-Ph	14.72 ± 1.0
15	I	N(CH3) CH2-Ph	1.02 ± 0.10

[a] K_i are means ± SEM of five determinations.
[b] 5-Deoxide derivatives.
[c] See ref.33.
[d] See Ref. 30.

the 8-NH-CH_2phenyl chain of compound **11** (K_i = 0.109 nM) in a linear manner, (**14**, 8-NH-CH_2CH_2phenyl–) or in a branched manner, (**15**, (8-N(CH_3)CH_2phenyl–) caused a 10–140-fold decrease in affinity although always in the nanomolar range: **14**, K_i = 14.72 nM and **15**, K_i = 1.02 nM. Thus the better influence of the 8-NH-CH_2phenyl group, even if modified, on receptor interaction than the 8-*O*-benzyl group[30] is evidenced; in fact, the affinity values of the 8-NH- derivatives are 3–10-fold higher than the 8-*O*-derivatives, as evidenced in a previous paper on derivatives bearing the NH-pyrrole moiety.[36]

Interesting information was obtained from the 5-*N*-deoxide derivatives **4R**, **7R** and **11R**. These compounds have comparable or better affinity values than their corresponding 5-oxide derivatives. Compare **4R**, K_i = 1.60 nM nM versus **4**, K_i = 4.66 nM, **7R**, K_i = 2.78 nM versus **7**, K_i = 2.58 nM and **11R**, K_i = 0.71 nM versus **11**, K_i = K_i = 0.109 nM. This trend indicates the secondary role of the N-oxide group in binding as already reported.[29,30]

Since we try to identify the chemical features of antihyperalgesic activity of compounds with a pyrazolo[5,1-*c*][1,2,4]benzotriazine core 3, 8-disubstituted, the in vivo tests were conducted on a sample of compounds representing all types of substitution. Thus, the new compounds **4**, **7**, **11** and **15** were chosen to evaluate the potential anxiolytic-like effects compared with those of diazepam and antagonized by flumazenil ($GABA_A$-R subtype antagonist). Then, the antihyperalgesic effect in different pain models was investigated. For these tests two already reported derivatives were considered, in this paper numbered **9**[33] and **10**(±)[30] since they are endowed with the 8-substituents suitable for our investigation: the 8-pentyloxy group in compound **9**[33] and the 8-(2-methyl-2-phenylethoxy) group in **10**(±).[30]

The effects on mouse anxiety of newly synthesized molecules, **4** and **7** (3–30 mg/kg po), **11** (3–10 mg/kg po), **15** and **9**,[33] **10**(±),[30] (10–30 mg/kg po), were studied using a light/dark box apparatus, Figure 1a and b.

In our experiments, compounds **4** (10–30 mg/kg po), **7**, **9**,[33] **10**(±)[30] and **15** (30 mg/kg po), **11** (10 mg/kg po), showed good anxiolytic-like effect with efficacy comparable to diazepam (standard reference). The anxiolytic-like effects exhibited were completely antagonized by flumazenil (at a dose of 100 mg/kg ip, a dose at which flumazenil was able to antagonize the anxiolytic effect of diazepam) suggesting that the anxiolytic-like effect of the newly synthesized compounds is exerted through the $GABA_A$ subtype receptors (through the benzodiazepine receptor).

Compounds **4**, **7**, **9**,[33] **10**(±),[30] **11** and **15** were tested on a pain model in which peripheral mono-neuropathy was produced in adult rats by loosely placing constrictive ligatures around the common sciatic nerve, according to the method described by Bennett and Xie.[37] The nociceptive threshold in the rat was determined with an analgesimeter according to the paw pressure test.[38] Figures 2a, b and c depict, for the sake of clarity, only graphics of the active compounds (**4**, **10**(±) and **11**); results for all compounds are reported in the Supplementary data.

Experiments were performed on rats submitted to the paw-pressure test 14 days after the operation since at this time a significant reduction in the pain threshold of the injured paw (dx) was observed. By contrast, in the contralateral paw the pain perception remained unchanged. Compounds **4** (10–600 mg/kg po), **10**(±) (10–30 mg/kg po) and **11** (3–30 mg/kg po) showed statistical antihyperalgesic activity on this model when compared with the CMC treated group, Figures 2a–c, respectively. The effect was observable 15, 30 and 45 min after administration for compounds **4** and **11** and up to 30 min for **10**(±). Compound **11** exhibited a higher potency since it exerted its protective effect starting from the dose of 3 mg/kg po while the first active dose for the other compounds, **4** and **10**(±), was 10 mg/kg po (see Figs. 2a–c). The above-mentioned compounds at the active doses did not modify the pain threshold in the contralateral, non-operated paw, demonstrating the lack of any analgesic effect.

Rats treated with **7**, **9** and **15** (30 mg/kg po) in the same painful condition did not exhibit any antihyperalgesic activity (data reported in Supplementary data).

Compounds **4**, **7**, **9**,[33] **10**(±),[30] **11** and **15** were also tested in the animal model of streptozotocin (STZ)-induced hyperalgesia which reproduces the pain caused by diabetic neuropathy in laboratory animals. STZ is particularly toxic for the insulin-producing beta cells of the pancreas in mammals and it drastically increases the serum glucose level. In diabetic patients hyperglycemia-induced oxidative stress has been considered the main cause of diabetic neuropathy.[39]Reactive oxygen species generated from an excess shunting of glucose, leads to the formation and activation of protein kinase C and consequently to nervous tissue damage and pain. Diabetic neuropathy can be due also to microvascular injury involving small blood vessels that supply nerves in addition to macrovascular conditions. It affects pain fibers, motor neurons and the autonomic nerve. The identification of new molecules endowed with activity against pain is very important since, despite advances in the understanding of the metabolic causes of neuropathy, treatment to reduce pain is limited.

The hyperalgesia induced by STZ and its reversal caused by the treatment with **4**, **10**(±),[30] and **11** (30 mg/kg po) 15, 30, and 45 min after their administration, is evidenced in the murine hot plate test and after a single injection, Figure 3.

The antihyperalgesic effect remained up to 60 min for compounds **10**(±) and **11** (see complete data reported in the Supplementary data). These molecules injected at the same doses were not able to increase the pain threshold in the absence of the STZ-induced painful condition. Derivatives **7**, **9** and **15** were devoid of any effect on the reduction of pain threshold provoked by STZ.

Compounds **4** and **11** (10–30 mg/kg po), **7**, **9**, **10**(±)and **15** (30–60 mg/kg po) were all unable to reduce the number of writhes induced by ip injection of a solution of acetic acid, confirming the lack of analgesic efficacy of these molecules, Table 3.

Protection from convulsions was evaluated in mice using pentylentetrazole (PTZ) as a chemical convulsant agent. All compounds **4**, **7**, **9**,[33] **10**(±),[30] **11** and (30 mg/kg po) and **15** (10 mg/kg po) were devoid of any effect on PTZ-shock whereas diazepam (1 mg/kg ip), the reference drug, completely protected against PTZ-induced shocks and convulsions, Table 4.

This result demonstrates that the mechanism of action responsible for their antihyperalgesic effect is not identifiable with anticonvulsant activity. This investigation was necessary because in the scientific literature the ability of antiepileptic drugs to relieve neuropathic painful conditions is widely reported.[40–43]

4. Conclusion

All the new compounds **3–8**, **11–15** were designed to optimize a previously published compound, **III** (chart 1) that showed the most promising effects in the peripheral mono-neuropathy pain model in adult rats. All new compounds were studied in vitro and compounds **4**, **7**, **9**,[33] **10**(±),[30] **11** and **15** were chosen for in vivo tests.

In regard to structure–affinity relationships, the simultaneous presence at position 8 of a –NH– moiety and at position 3 of iodine atom, improve binding affinity, reaching a sub-nanomolar range, compared to the 3-methyl derivatives. This indicates that it is very important for receptor protein interaction the presence of a lipophilic group (iodine) and of a group that engages a hydrogen bond (–NH–), as also evidenced in previous paper.[30,36] In fact, the optimization of the compound **III**[30] was realized with the synthesis of compound **11**, 3-iodo-8-benzylaminopyrazolo[5,1-*c*][1,2,4] benzotriazine 5-oxide, that stands out for its very high affinity value

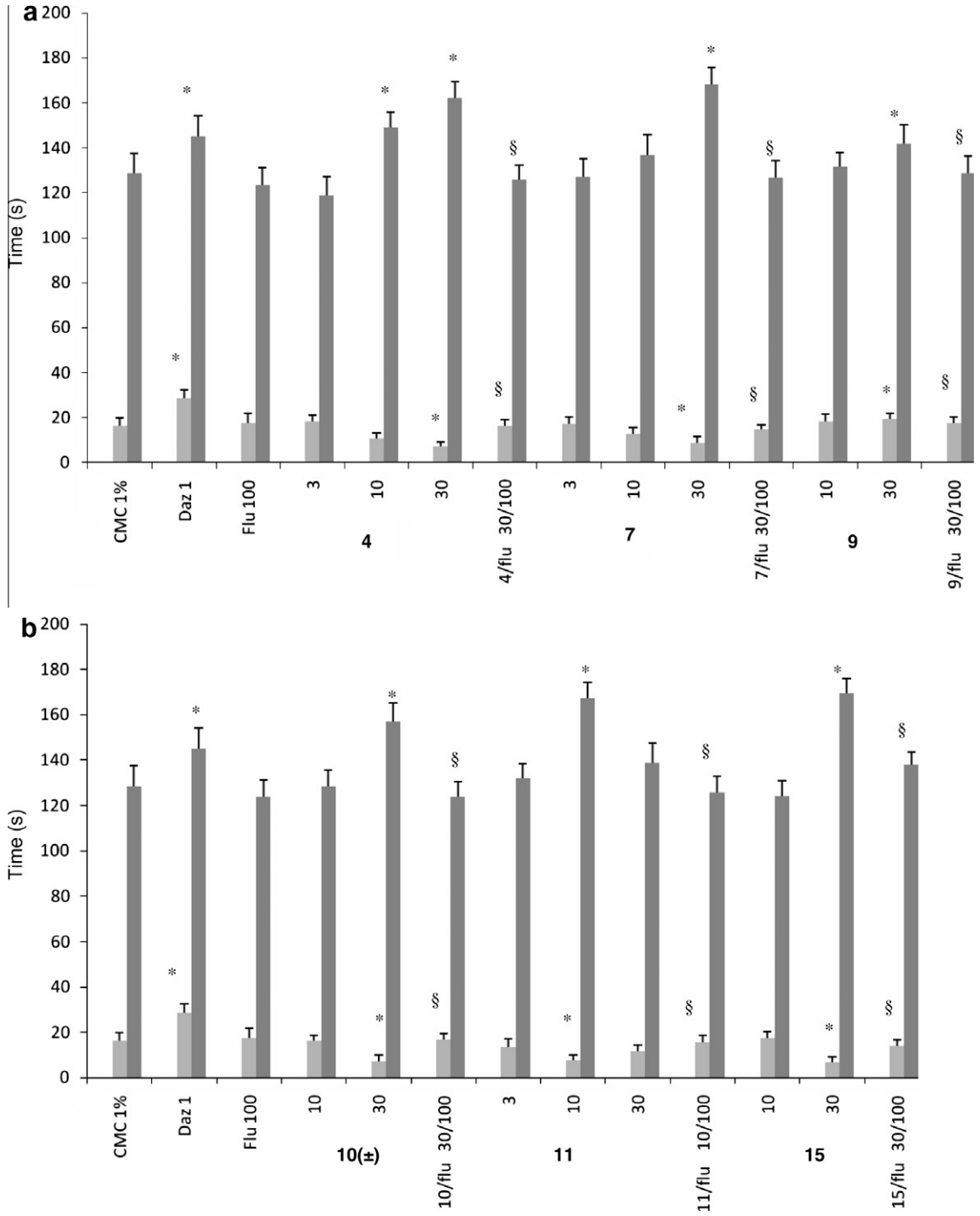


Figure 1. (a and b) Anxiolytic like-effect of compounds **4**, **7** and **9** (panel 1a) and **10**(±), **11** and **15** (panel 1b) in comparison with diazepam (Daz, i.p.) on mouse light-dark box test. The first column represents the number of transfers in 5 min, and the second column represents the time spent in light. Compound (p.o.) and diazepam were administered 30 min and flumazenil (flu, i.p.) before the test. Each column represents the means ± SEM of 8 mice. $^{*}P$ <0.01versus saline treated mice. $^{§}P$ <0.01 vs the corresponding compound-treated mice. For sake of clarity compounds were divided in two panel.

(K_i = 0.109 nM) also with respect to its corresponding 3-methyl derivative **7** (K_i = 2.58 nM).

In the animal pain models (peripheral mono-neuropathy, hyperalgesia STZ-induced) only compounds **4**, **10**(±),[30] and **11** with selective anxiolytic profile, showed antihyperalgesic effects at the dose of 10–30 mg/kg. Again compound **11**, emerges because exhibits a higher potency since it exerted its protective effect at 3 mg/kg. It is important to note that compounds **4**, **10**(±) and **11** exerted their antihyperalgesic effect after a single injection while several substances, such as aldose reductase inhibitors, antioxidants (including α-lipoic acid, dimethylthiourea, the xanthine oxidase inhibitor allopurinol and taurine), PKC- or PARP-inhibitors, prevent thermal hyperalgesia in STZ-diabetic rodents only after repeated administration.[44] The efficacy of **4**, **10**(±) and **11** is comparable with that of lacosamide or kinin B1 receptor antagonists that produced full reversal of thermal hyperalgesia.[45] Moreover, **4**, **10**(±) and **11** show the antihyperalgesic effect at a dosage comparable to that of other described subtype-selective benzodiazepine-site ligands endowed with anxiolytic-like effect.[26]

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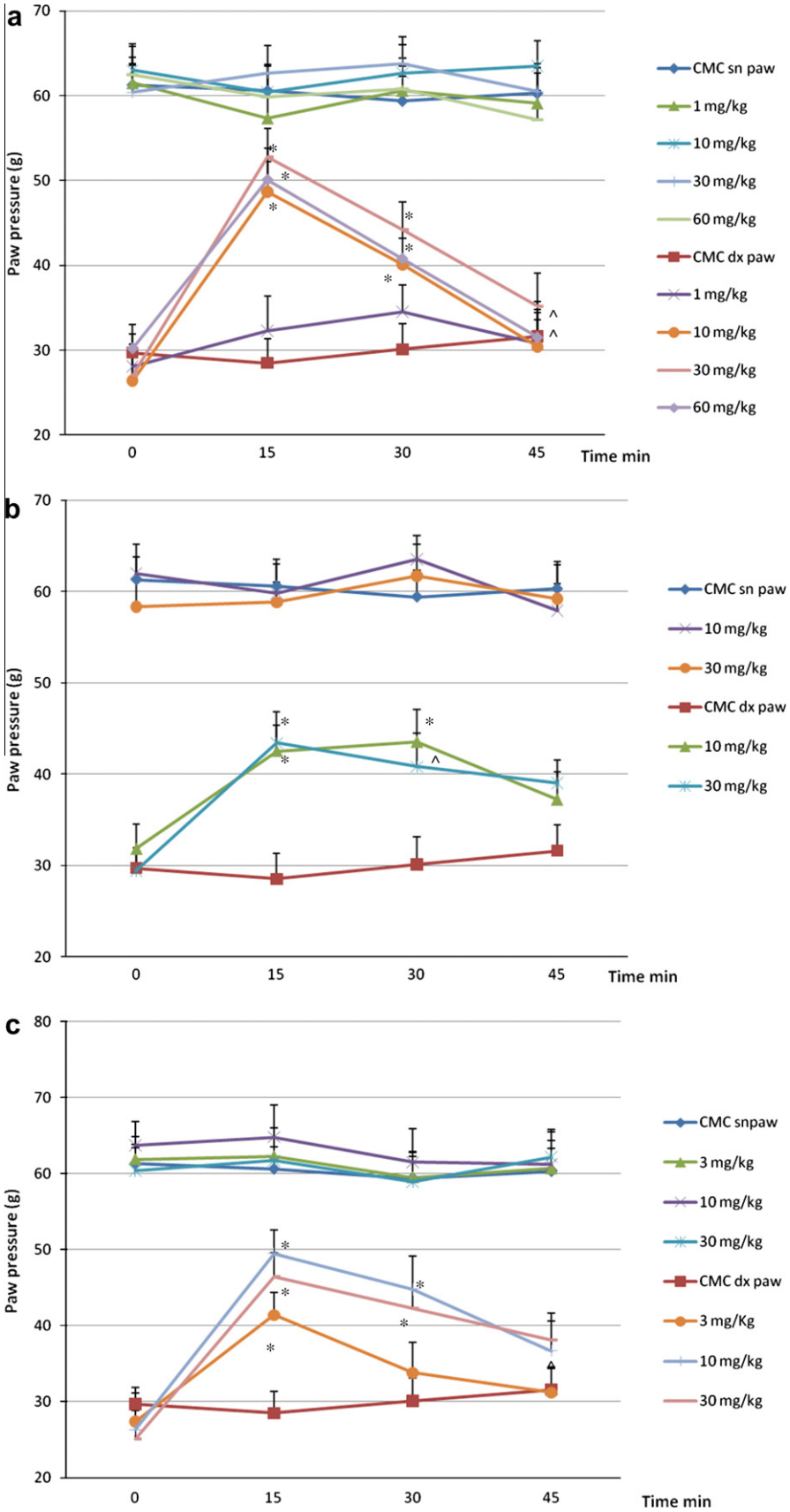

Figure 2. (a) Effect of **4** (1–60 mg/kg) in a rat model of mononeuropathy dx evaluated in the paw-pressure test. *P <0.01, ^P <0.05.There were at least 8 rats per group. (b) Effect of **10**(±) (10–30 mg/kg) in a rat model of mononeuropathy dx evaluated in the paw-pressure test. *P <0.01, ^P <0.05. There were at least four rats per group. (c) Effect of **11** (3-30 mg/kg) in a rat model of mononeuropathy dx evaluated in the paw-pressure test. *P <0.01, ^P <0.05. There were at least four rats per group.

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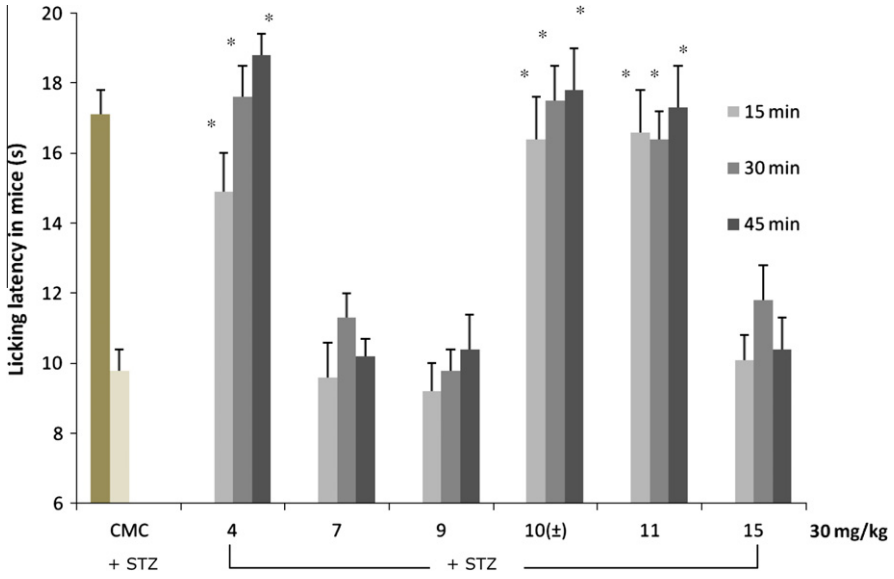

Figure 3. Streptozotocin (STZ) 200 mg/kg i.p. was dissolved in citrate buffer and injected 21 days before experiment. Each value represents the mean of at least 10 mice.*P<0.01 versus STZ-treated mice.

Table 3
Effect of compounds **4**, **7**, **9**, **10**, **11**, **15** in the mouse abdominal constriction test (acetic acid 0.6%)

Treatment[a]		*n*[b]	Number of writhes
Compound	mg/kg p.o.		
CMC 1%		15	32.4 ± 2.5
4	10	9	31.2 ± 3.5
	30	10	27.3 ± 3.2
7	30	10	29.1 ± 2.4
	60	10	27.4 ± 2.6
9	30	10	30.7 ± 2.7
	60	10	28.5 ± 2.8
10	30	8	31.6 ± 3.1
	60	9	27.3 ± 2.6
11	10	10	26.3 ± 3.1
	30	8	26.1 ± 2.5
15	30	9	28.8 ± 2.9
	60	8	27.5 ± 3.0

[a] All drugs were administered 30 min before test.
[b] Number of mice.

Table 4
Anticonvulsant activity of compounds **4**, **7**, **9**, **10**(**±**), **11**, **15**

Treatment[a]		% Protection on PTZ-induced[b] convulsions
Compound	mg/kg po	
4	30	10
7	30	10
9	30	0
10(**±**)	30	0
11	30	10
15	10	10
Diazepam	1	100*

[a] Treatment with new compounds and diazepam (ip) was performed 30 min before the test.
[b] Pentylenetetrazole (PTZ) 90 mg kg^{-1} ip Each value represents the mean of 10 mice.

These findings represent a starting point for the development of a novel class of ligands at $GABA_A$ subtype receptors with antihyperalgesic effect since the synthesized compounds have anxiolytic-like effect and have the potential to be therapeutic agents for the relief of neuropathic pain, while being devoid of undesirable effects. This dual action on emotion end pain may be particularly useful therapeutically. Additional work is in progress to gain more information on the receptor interaction (using our pharmacophore ADLR procedure[30]) and to study in deep pharmacological aspects and the why some compounds with anxiolytic-like effect (**7**, **9** and **15**) have not antihyperalgesic effect.

5. Experimental section

5.1. Chemistry

Melting points were determined with a Gallenkamp apparatus and were uncorrected. Silica gel plates (Merk F_{254}) and silica gel 60 (Merk 70–230 mesh) were used for analytical and column chromatography, respectively. The structures of all compounds were supported by their IR spectra (KBr pellets in nujol mulls, Perkin–Elmer 1420 spectrophotometer) and ^{1}H NMR data (measured with a Bruker 400 MHz). Chemical shifts were expressed in δ ppm, using DMSO-d_6 or $CDCl_3$ as solvent. The chemical and physical data of new compounds are shown in Tables 1; all new compounds possess a purity $\geqslant$95%: microanalyses were performed with a Perkin–Elmer 260 analyzer for C, H, N. Mass spectra (*m/z*) were recorded on a ESI-MS quadrupole (Varian 1200L) system, in positive ion mode, infusing a 10 mg/L solution of each analyte, dissolved in a mixture of mQ H_2O:acetonitrile 1:1 v/v.

5.1.1. Sodium 2-cyanoprop-1-en-1-olate

A solution of propionitrile (108 mmol) and ethylformate (118 mmol) in anhydrous diethyl ether (20 mL) was added dropwise to a suspension of sodium hydride 50% (108 mmol) in anhydrous diethyl ether (250 mL) while temperature was maintained below 30 °C. The reaction was stirred for 12–16 h and, after cooling, the sodium salt was filtered and washed with anhydrous diethyl ether solid. The sodium salt was immediately used for the next reaction. IR ν cm^{-1} 2214. Anal C, H, N.

5.1.2. 1-(2-nitro-5-chlorophenyl)-2*H*-4-methyl-5-iminopyrazole (1)

A suspension of sodium 2-cyanoprop-1-en-1-olate (7.0 mmol) and ethanol (20 mL) was acidified with hydrochloride acid conc. up to pH 2–3. The suspension was stirred and added of a solution

of 2-nitro-5-chlorophenylhydrazine[46] (1.0 mmol) in ethanol (20 mL); the reaction was stirred at refluxing temperature, for 2 h, and monitored by TLC until starting material disappearing. The formed sodium chloride was filtered off and the ethanol solution was evaporated in vacuo. The crude dark red product was washed by ether and characterized (870 mg, 35% yield). TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1}3303; ^1H NMR (DMSO-d_6) δ 11.10 (s, 1H, =NH exchang.); 8.01 (br s, 1H, H-3); 7.80 (m, 2H, H-3'and H-6'); 6.90 (dd, J = 2.0, 9.0 Hz, 1H, H-4'); 4.10 (s, 1H, NH exchang.); 1.71 (s, 3H, CH_3). Anal C, H, N.

5.1.3. 1-(2-nitro-5-chlorophenyl)-4-methyl-5-aminopyrazole (2)

A suspension of sodium 2-cyanoprop-1-en-1-olate (7.0 mmol) and ethanol (20 mL) was acidified with hydrochloride acid conc. up to pH 2–3. The suspension was stirred and added of a solution of 2-nitro-5-chlorophenylhydrazine[46] (1.0 mmol) in ethanol (20 mL); the reaction was stirred at refluxing temperature, for 10 h, and the imino derivative (**1**) was converted in the corresponding 5-aminopyrazole **2**. By TLC monitoring is possible to evidence the spot of 5-aminopyrazole (**2**) at lower R_f than 5-iminopyrazole (**1**); when the reaction was finished the ethanol solution was evaporated and the final precipitate recrystallized with ethanol (1.05 g, 42% yield). Yellow crystals; TLC eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3299; ^1H NMR (DMSO-d_6) δ 8.10 (d, J = 9.1 Hz, 1H, H-3'); 7.85 (d, J = 1.9 Hz, 1H, H-6'); 7.76 (dd, J = 1.9, 9.1 Hz, 1H, H-4'); 7.38 (s, 1H, H-3); 3.80 (br s, 2H, NH_2 exchang.); 1.85 (s, 3H, CH_3). Anal C, H, N.

5.1.4. 3-methyl-8-cloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide 3

The 5-iminopyrazole (**1**) or the 5-aminopyrazole (**2**) (1.0 mmol) dissolved in 1 mL of diglyme (diethylene glycol dimethyl ether) and 30 mL of 10% sodium hydroxide solution was added. The reaction was maintained at 50 °C under stirring for 12 h. The final suspension was filtered and the crude yellow product was recrystallized by suitable solvent (176 mg, 75% yield). Yellow crystals; TLC eluent: isopropyl ether/ciclohexane 8:3 v/v; IR ν cm^{-1} 1580; ^1H NMR (DMSO-d_6) δ 8.41 (d, J = 9.2 Hz, 1H, H-6); 8.31 (d, J = 2.4 Hz, 1H, H-9); 8.17 (s, 1H, H-2); 7.72 (dd, J = 2.4, 9.2 Hz, 1H, H-7); 2.35 (s, 3H, CH_3). Anal C, H, N.

5.1.5. General procedure for the synthesis of 4–6(±)

The reaction was carried out in 10 mL of dichloromethane to which was added the starting material **3** (0.3 mmol), 5 mL of 40% sodium hydroxide solution, 0.5 mmol of tetrabutylammonium bromide, and the suitable alcohol in large excess (ratio with starting material 1:15) under vigorous stirring (temperature and time specified for each compound). The reaction was monitored by TLC, and when the starting material disappeared, the organic layer was separated and the aqueous layer extracted twice with 10 mL of dichloromethane. The combined organic extracts were evaporated and the residue was recovered with isopropil ether and then recrystallized by suitable solvent.

5.1.6. 3-methyl-8-benzyloxypyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (4)

From **3** and benzyl alcohol, at 50 °C for 32 h; 52 mg, 57% yield. Yellow crystals; TLC eluent: ciclohexane/ethyl acetate 2:1 v/v; ^1H NMR (DMSO-d_6) δ 8.35 (d, J = 9.6 Hz, 1H, H-6); 8.13 (s, 1H, H-2); 7.74 (d, J = 2.1 Hz, 1H, H-9); 7.53 (d, J = 7.6 Hz, 2H, Ph); 7.42 (t, J = 7.6 Hz, 2H, Ph); 7.39 (t, J = 7.6 Hz, 1H, Ph); 7.32 (dd, J = 2.1, 9.6 Hz, 1H, H-7); 5.42 (s, 2H, CH_2); 2.26 (s, 3H, CH_3). Anal C, H, N.

5.1.7. 3-methyl-8-(2-methoxybenzyloxy)pyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (5)

From **3** and 2-methoxybenzyl alcohol, at 40 °C for 24 h; 52 mg, 52% yield. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^1H NMR (DMSO-d_6) δ 8.33 (d, J = 9.0 Hz, 1H, H-6); 8.13 (s, 1H, H-2); 7.74 (d, J = 2.8 Hz, 1H, H-9); 7.48 (d, J = 7.2 Hz, 1H, H-6' Ph); 7.39 (t, J = 7.2 Hz, 1H, H-4'Ph); 7.30 (dd, J = 2.8, 9.0 Hz, 1H, H-7); 7.10 (d, J = 7.2 Hz, 1H, H-3' Ph); 7.00 (t, J = 7.2 Hz, 1H, H-5' Ph); 5.35 (s, 2H, CH_2); 3.88 (s, 3H, OCH_3); 2.26 (s, 3H, CH_3). Anal C, H, N.

5.1.8. 3-methyl-8-(2-methyl-2-phenylethoxy)pyrazolo[5,1-c]-[1,2,4]benzotriazine 5-oxide (6(±))

From **3** and 2-phenylpropan-1-ol at 50 °C for 32 h; 34 mg, 34% yield. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; ^1H NMR (DMSO-d_6) $\delta\delta$ 8.30 (d, J = 9.6 Hz, 1H, H-6); 8.12 (s, 1H, H-2); 7.61 (d, J = 2.8 Hz, 1H, H-9); 7.28–7.34 (m, 4H, Ph); 7.21 (m, 2H, H-7 and Ph); 5.08 (m, 2H, CH_2); 3.02 (m, 1H, CH); 2.25 (s, 3H, 3-CH_3); 1.35 (s, 3H, CH_3). Anal C, H, N.

5.1.9. General procedure for the synthesis of 7–8, 11–15

A solution of the starting material **3** (0.35 mmol) or 3-iodo-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide,[34] in ethanol (5.0 mL) was added of an excess of suitable amine (1.0 mL) and refluxed under vigorous stirring (time specified for each compound). The reaction was monitored by TLC, and when the starting material disappeared, the final precipitate was filtered, or in case of a solution, ice was added to obtain a precipitate; then the crude residue was washed with isopropyl ether and recrystallized by suitable solvent.

5.1.10. 3-methyl-8-benzylaminopyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide (7)

From **3** and benzyl amine for 48 h; 42.7 mg, 40% yield. Yellow crystals; TLC and chromatography column eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3271; ^1H NMR (DMSO-d_6) δ 8.20 (m, 1H, NH, exchang.); 8.11 (d, J = 8.9 Hz, 1H, H-6); 8.01 (s, 1H, H-2); 7.42–7.38 (m, 4H, Ph); 7.28 (m, 1H, Ph); 7.04 (dd, J = 2.7, 8.9 Hz, 1H, H-7); 6.80 (d, 2.7 Hz, 1H, H-9); 4.55 (d, J = 5.2 Hz, 2H, CH_2); 2.26 (s, 3H, CH_3). Anal C, H, N.

5.1.11. 3-methyl-8-(2-methoxybenzylamino)pyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (8)

From **3** and 2-methoxybenzylamine for 56 h; 64.5 mg, 55% yield. Dark yellow crystals; TLC and chromatography column eluent: toluene/ethyl acetate 8:2 v/v; IR ν cm^{-1} 3271; ^1H NMR (DMSO-d_6) δ 8.11 (d, J = 9.0 Hz, 1H, H-6); 8.03 (m, 1H, NH, exchang.); 8.00 (s, 1H, H-2); 7.27 (m, 2H, H-4 and H-6 Ph); 7.09 (m, 2H, H-9 and H-3 Ph); 6.97 (dd, J = 2.8, 9.0 Hz, 1H, H-7); 6.92 (t, J = 6.9 Hz, 1H, H-5 Ph); 4.45 (d, J = 5.2 Hz, 2H, CH_2); 3.90 (s, 3H, OCH_3); 2.20 (s, 3H, CH_3). Anal C, H, N.

5.1.12. 3-iodo-8-benzylaminopyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (11)

From 3-iodo-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide,[34] and benzyl amine for 36 h; 119.7 mg, 82% yield. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1}3270; ^1H NMR (DMSO-d_6)) δ 8.32 (m, 1H, NH, exchang.); 8.22 (s, 1H, H-2); 8.12 (d, J = 8.9 Hz, 1H, H-6); 7.40–7.36 (m, 4H, Ph); 7.27 (m, 1H, Ph); 7.02 (m, 2H, H-7 and H-9); 4.52 (d, J = 5.2 Hz, 2H, CH_2). Anal C, H, N.

5.1.13. 3-iodo-8-(2-methoxybenzylamino)pyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (12)

From 3-iodo-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide,[34] and 2-methoxybenzylamine for 16 hours; 99.8 mg, 64% yield. Orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1}3302; ^1H NMR (DMSO-d_6) δ 8.22 (s, 1H, H-2); 8.18 (m, 1H, NH, exchang.); 8.11 (d, J = 9.1 Hz, 1H, H-6); 7.29 (m, 2H, H-4 and H-6 Ph); 7.09 (m, 2H, H-9 and H-3 Ph); 7.03 (dd, J = 2.6, 9.1 Hz, 1H, H-7); 6.92 (t, J = 7.0 Hz, 1H, H-5 Ph); 4.45 (d, J = 5.2 Hz, 2H, CH_2); 3.90 (s, 3H, OCH_3). Anal C, H, N.

5.1.14. 3-iodo-8-(4-chlorobenzylamino)pyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (13)

From 3-iodo-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide,[34] in DMF (2.0 mL) and 4-chlorobenzylamine for 48 hours; 94.8 mg, 60% yield. Orange crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3304; ^{1}H NMR (DMSO-d_6)) δ 8.32 (m, 1H, NH, exchang.); 8.27 (s, 1H, H-2); 8.12 (d, J = 9.0 Hz, 1H, H-6); 7.43 (m, 4H, Ph); 7.02 (m, 2H, H-7 and H-9); 4.55 (d, $J = J$ = 5.2 Hz, 2H, CH_2). Anal C, H, N.

5.1.15. 3-iodo-8-(2-phenylethylamino)pyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (14)

From 3-iodo-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide,[34] and phenylethylamine for 10 hours; 120.7 mg, 80% yield. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3303; ^{1}H NMR (DMSO-d_6) δ 8.27 (s, 1H, H-2); 8.12 (d, J = 8.9 Hz, 1H, H-6); 7.88 (m, 1H, NH, exchang.); 7.33 (m, 4H, Ph); 7.24 (m, 1H, Ph); 7.08 (d, J = 2.7 Hz, 1H, H-9); 6.98 (dd J = 2.7, 8.9 Hz, 1H, H-7); 3.53 (t, J = 5.0 Hz, 2H, CH_2NH); 2.96 (t, J = 5.0 Hz, 2H, CH_2Ph). Anal C, H, N.

5.1.16. 3-iodo-8-(benzyl(methyl)amino)pyrazolo[5,1-c][1,2,4]-benzotriazine 5-oxide (15)

From 3-iodo-8-chloropyrazolo[5,1-c][1,2,4]benzotriazine 5-oxide,[34] and N-methylbenzylamine for 10 hours; 84.5 mg, 56% yield. Yellow crystals; TLC eluent: toluene/ethyl acetate/acetic acid 8:2:1 v/v/v; IR ν cm^{-1} 3303; ^{1}H NMR^{1}H NMR (DMSO-d_6) δ 8.25 (s, 1H, H-2); 8.18 (d, J = 9.1 Hz, 1H, H-6); 7.37 (m, 2H, H-2 and H-6 Ph); 7.26 (m, 3H, H-3, H-4 and H-5 Ph); 7.16 (m, 2H, H-6 and H-9); 4.90 (s, 2H, CH_2); 3.33 (s, 3H, N-CH_3). Anal C, H, N.

5.1.17. General procedure for the synthesis of 4R, 7R and 11R

A solution of suitable 5-oxide derivative **4**, **7** and **11** (0.3 mmol) in acetic acid (10 mL) was magnetically stirred at room temperature. A large excess of zinc dust (2.0 mmol in three portions) and 2 × 5 mL of acetic acid were added. After 15 min the reaction was stopped, since the starting material disappeared (evaluated by TLC, eluent ethyl acetate/ petroleum benzine 2:1 v/v) and the zinc residue was filtered off. The solution was evaporated in vacuo and the final sugary residue was treated with ice/water. In case of derivative **4R** the aqueous solution was extracted with ethyl acetate and, after anhydrification with sodium sulfate anhydrous, evaporated. The residue was recovered with di-isopropyl ether, filtered and recrystallized by suitable solvent. In case of derivatives **7R** and **11R** the aqueous solution was made slightly alkaline (pH 7–8) to permit the final compounds to precipitate. Also in this case, the precipitate was filtered and recrystallized by suitable solvent.

5.1.18. 3-methyl-8-benzyloxypyrazolo[5,1-c][1,2,4]-benzotriazine (4R)

From **4**; 59.2 mg, 68% yield. Yellow crystals; TLC eluent: ethyl acetate/petroleum benzine 2:1 v/v; ^{1}H NMR ($CDCl_3$) δ 8.50 (d, J = 9.2 Hz, 1H, H-6); 8.05 (s, 1H, H-2); 8.38 (d, J = 2.4 Hz, 1H, H-9); 7.52 (d, $J = J$ = 7.2 Hz, 2H, Ph); 7.44 (t, J = 7.2 Hz, 2H, Ph); 7.39 (t, J = 7.2 Hz, 1H, Ph); 7.33 (dd, J = 2.4, 9.2 Hz, 1H, H-7); 5.32 (s, 2H, CH_2); 2.69 (s, 3H, CH_3); ESI-MS: found m/z 291.1 $[M+H]^+$. Anal C, H, N.

5.1.19. 3-methyl-8-benzylaminopyrazolo[5,1-c][1,2,4]-benzotriazine (7R)

From **7**; 62.5 mg, 72% yield. Pale orange crystals; TLC eluent: ethyl acetate/petroleum benzine 2:1 v/v; IR ν cm^1 3270; ^{1}H NMR ($CDCl_3$) δ 8.29 (d, J = 8.8 Hz, 1H, H-6); 7.89 (s, 1H, H-2); 7.41 (m, 4H, Ph); 7.37 (m, 1H, Ph); 7.32 (d, J = 2.4 Hz, 1H, H-9); 6.93 (dd, J = 2.4, 8.8 Hz, 1H, H-7); 5.11 (m, 1H, NH, exchang.); 4.57 (d, J = 5.2 Hz, 2H, CH_2); 2.65 (s, 3H, CH_3); ESI-MS: found m/z 290.2 $[M+H]^+$. Anal C, H, N.

5.1.20. 3-iodo-8-benzylaminopyrazolo[5,1-c][1,2,4]-benzotriazine (11R)

From **11**; 66.2 mg, 55% yield. Yellow crystals; TLC eluent: ethyl acetate/petroleum benzine 2:1 v/v; IR ν cm^1 3337; ^{1}H NMR (DMSO-d_6) δ 8.45 (m, 1H, NH, exchang.); 8.35 (s, 1H, H-2); 8.25 (d, J = 8.9 Hz, 1H, H-6); 7.48–7.36 (m, 4H, Ph); 7.33 (m, 1H, Ph); 7.28 (d, J = 2.3 Hz, 1H, H-9); 7.22 (dd, J = 2.3, 8.9 Hz, 1H, H-7); 4.60 (d, 2H, CH_2); ESI-MS: found m/z 402.1 $[M+H]^+$. Anal C, H, N.

5.2. Radioligand binding assay

[^{3}H]Ro15–1788 (specific activity 78.8 Ci/mmol) was obtained from Perkin Elmer. All the other chemicals, which were of reagent grade, were obtained from commercial suppliers.

Bovine cerebral cortex membranes were prepared as previously described.[47,48] The membrane preparations were diluted with 50 mM tris-citrate buffer pH 7.4, and used in the binding assay. Protein concentration was assayed using the method of Lowry et al.[49] [^{3}H]Ro 15–1788 binding studies were performed as previously reported.[50] At least six different concentrations of each compound were used. The data of n = 5 experiments carried out in triplicate were analyzed by means of an iterative curve-fitting procedure (program Prism, GraphPad, San Diego, CA), which provided IC_{50}, K_i, and SEM values for tested compounds, the K_i values being calculated from the Cheng and Prusoff equation.[51]

5.3. Pharmacological methods

Animals: Male Swiss albino mice (23–30 g) and male Sprague–Dawley rats from Harlan- (Varese) breeding farm were used. Fifteen mice or four rats were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatisation. The animals were kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m., with food and water ad libitum. All experiments were carried out according to the guidelines of the European Community Council.

5.3.1. Pentylenetetrazole (PTZ)-induced seizure

PTZ (90 mg/kg s.c.) was injected 30 min after the administration of drugs. The frequency of the occurrence of clonic generalized convulsions was noted over a period of 30 min.

5.3.2. Mouse light/dark box test

The apparatus (50 cm long, 20 cm wide, and 20 cm high) consisted of two equal acrylic compartments, one dark and one light, illuminated by a 60 W bulb lamp and separated by a divider with a 10 × 3-cm opening at floor level. Each mouse was tested by placing it in the center of the lighted area, facing away from the dark one, and allowing it to explore the novel environment for 5 min. The number of transfers from one compartment to the other and the time spent in the illuminated side were measured. This test exploited the conflict between the animal's tendency to explore a new environment and its fear of bright light.

5.3.3. Hot plate test

The method adopted was described by O'Callaghan and Holzman.[52] Mice were placed inside a stainless steel container, thermostatically set at 52.5 ± 0.1 °C in a precision water-bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s), were measured with a stop-watch before i.c.v. injections and at regular intervals (15 min) up to a maximum of 60 min after treatment (cut-off) in order to prevent tissue damage. The endpoint used was the licking of the fore or hind paws. Antinociception was seen as increased latencies to the responses evaluated, while increased nociception was seen by shorter latencies. Those mice scoring below 12 and

over 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted.

5.3.4. Abdominal constriction test

Mice were injected i.p. with a 0.6% solution of acetic acid (10 ml kg^{-1}), according to Koster.[53] The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

5.3.5. Paw pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton.[38]The instrument exerts a force which is applied at a costant rate (32 g per second) with a cone-shaped pusher on the upper surface of the rat hind paw. The force is continuously monitored by a pointer moving along a linear scale. The pain threshold is given by the force which induces the first struggling from the rat. Pretested rats which scored below 40 g or over 75 g during the test before drug administration (25%) were rejected. An arbitrary cut off value of 250 g was adopted.

5.3.6. Chronic Constriction Injury (CCI)

A peripheral mono neuropathy was produced in adult rats by placing loosely constrictive ligatures around the common sciatic nerve according to the method described by Bennett.[37] Rats were anaesthetised with chloral hydrate. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to sciatica's tri furcation, about 1 cm of the nerve was freed of adhering tissue and four ligatures (3/0 silk tread) were tied loosely around it with about 1 mm spacing. The length of the nerve thus affected was 4–5 mm long. Great care was taken to tie the ligatures such that the diameter of the nerve was seen to be just barely constricted when viewed with 40x magnification. In every animal, an identical dissection was performed on the opposite side except that the sciatic nerve was not ligated. The left paw was untouched.

5.3.7. Induction of diabetes (streptozotocin Treatment)

Mice were injected intravenously in the tail with 200 mg kg^{-1} of streptozotocin (STZ) prepared in saline adjusted to pH 4.5 in 0.1 N citrate buffer. Non-diabetic control mice were injected with the vehicle alone. STZ solutions were freshly prepared due to the limited stability of the compound. Rashid & Ueda,[54] in a set of preliminary control experiments, measured spectro- photometrically serum glucose level at 7, 14, and 21 days after streptozotocin treatment. They found that it was consistent with a diabetic level (above 300 mg/dl) throughout the periods. The serum glucose level was measured by glucose oxidase method from blood samples. The animals were found to develop significant thermal hyperalgesia at the 3rd week after streptozotocin treatment. In the pre-test performed in the test day those mice scoring over 20 s in hot plate test were rejected. Test was performed 21 days post streptozotocin treatment at 30, 60, 90 and 120 min after the last injection of test compounds.

5.4. Drugs

Diazepam (Valium 10–Roche), flumazenil (Roche), pentylenetetrazole (PTZ) (Sigma), zolpidem (Tocris) were used. All drugs except PTZ were suspended in 1% carboxymethylcellulose sodium salt and sonicated immediately before use. PTZ was dissolved in isotonic (NaCl 0.9%) saline solution and injected s.c. All benzodiazepine receptor ligands were administered by po route, except for flumazenil which was administered i.p. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml/kg by the po, i.p. or s.c. routes.

5.5. Statistical analysis

All experimental results are given as the mean ± S.E.M. Analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) procedure for post-hoc comparison, was used to verify significance between two means. Data were analysed with the StatView software for the Macintosh (1992). *P* values of less than 0.05 were considered significant.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.047.

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