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Identification of a new pyrazolo[1,5-a]quinazoline ligand highly affine to γ-aminobutyric type A ($GABA_A$) receptor subtype with anxiolytic-like and antihyperalgesic activity

Gabriella Guerrini,[a*] Giovanna Ciciani,[a] Letizia Crocetti,[a] Simona Daniele,[c] Carla Ghelardini,[b] Maria Paola Giovannoni,[a] Antonella Iacovone,[a] Lorenzo Di Cesare Mannelli,[b] Claudia Martini,[c] Claudia Vergelli[a]

[a]Dipartimento NEUROFARBA, sezione Farmaceutica e Nutraceutica, Università degli Studi di Firenze, Italy

[b]Dipartimento NEUROFARBA, sezione Farmacologia, Università degli Studi di Firenze, Italy

[c]Dipartimento FARMACIA, Università degli Studi di Pisa, Italy

*Corresponding author: Guerrini Gabriella, Dipartimento NEUROFARBA, sezione Farmaceutica e Nutraceutica, Università degli Studi di Firenze, Italy, Via Ugo Schiff, 6, Sesto Fiorentino Firenze, 50019; tel. +390554573766; e-mail: gabriella.guerrini@unifi.it

Abstract.

Compounds that can act on $GABA_A$ receptor subtype in selective manner, without the side effects of classical benzodiazepine ligands, represent promising therapeutic tools in neurological disorder, as well as for relief of pain or in comorbidity of anxiety states and depression. Continuing our research on $GABA_A$ receptor subtype ligands, here is reported the synthesis of a series of pyrazolo[1,5-a]quinazoline 3 and/or 8 substituted as 5-deaza analogues of previous reported pyrazolo[5,1-c][1,2,4]benzotriazine, already identified as selective $GABA_A$ receptor subtype ligands endowed with anxiolytic-like and antihyperalgesic action or enhancer cognition. Between the new compounds stands out **12b** for its high affinity value (K_i = 0.27 nM) and for its anxiolytic-like and ability to relieve neuropathic painful conditions evaluated in CCI and STZ murine model.

Introduction

The neurotransmitter γ-aminobutyric acid (GABA) and γ-aminobutyric acid type A receptors ($GABA_A$-Rs) play a key role within the central nervous system (CNS) being involved in a variety of disorder such as schizophrenia, anxiety and epilepsy, as well as in cognition, learning, memory and pain process.

$GABA_A$-Rs are heteropentameric ligand-gated ion channels formed by the combination of different subunits belonging to 8 families (α, β, γ, δ, ε, θ, π, ρ).[1] Briefly, in the CNS the majority of $GABA_A$-Rs are composed of α, β and γ subunits with subunit stoichiometry 2α:2β:1γ and γ-α-β-α-β arrangement, clockwise when viewed from the extracellular side.[2] However the possibility of different stoichiometry and arrangements (3α:1β:1γ and 2α:1β:2γ; γ-α-α-β-α and γ-α-γ-β-α counter-clockwise) for the most abundant αβγ subtypes and functional receptors was, recently, evidenced.[3] Inside the pentamer, the high affinity allosteric site of 'benzodiazepine' (BDZ) located in the extracellular α+/γ- interface is well-documented, and the affinity/efficacy of ligands depends on the type of α subunit (α1-6).[1] The existence of a low affinity benzodiazepine binding site located in the extracellular α+/β- interface, the homologous position at the α+/γ- interface, activate by micromolar concentrations of classical benzodiazepine receptor ligands (diazepam, 2-p-methoxyphenylpyrazolo[4,3-c]quinolin-3(5H)-one CGS9895), has been hypothesized[4,5] and recently confirmed[6–9] even if its role is currently under study. Moreover a third low affinity diazepam site, flumazenil insensitive, has been described as probably located in the second transmembrane domain (TM2) of receptor protein and it seems to be involved in the anesthetic property of diazepam.[8,10,11]

Extensive efforts have proven that the pharmacological functions of benzodiazepine site as part of the $GABA_A$ receptor (also $GABA_A$-R α1-3, 5 subtype or the high affinity allosteric site of BDZ), depend on the type of α subunit and these aspects have been deeply elucidated with genetically modified mice.[12] Thus, the α1 subunit mediates the anticonvulsant and sedative action, anterograde

amnesia and dependence liability of diazepam (the prototype of BDZ receptor ligands); the α2/α3 subunits are responsible of the anxiolityc-like effect while the α5 subunit is involved in cognition learning and memory.[13] In addition to these 'well-known roles' of α1-3,5-$GABA_A$ receptor subtypes, new and intriguing actions were evidenced for α2/α3- and for α5-$GABA_A$-R subtypes. In particular the evidenced relationship between α2/α3-subtypes and analgesic activity of diazepam[13] led to the synthesis of ligands with selective anxiolytic-like and anti-hyperalgesic action without sedation, motor impairment or tolerance development.[14–19] Regarding the α5-$GABA_A$-R subtypes, mainly localized in the hippocampus,[12] the role in the restoring memory deficits has been strongly evidenced and starting from the first negative allosteric modulator (NAM, also known as inverse agonist), ethyl (S)-11,12,13,13a-tetrahydro-7-methoxy-9-oxo-9H-imidazo[1,5-a]pyrrolo[2,1-c][1, 4]benzodiazepine-1-carboxylate (L-665708 or FG8094), many ligands have been synthesized.[20–22] An α5-$GABA_A$-Rs NAM might be able to reverse the cognitive deficit in various disorder including Down Syndrome (DS), Autism Spectrum Disorder (ASD), stroke, schizophrenia and dementia-related conditions.[12,23] However, since the α5-subunit is also present in extrasynaptic $GABA_A$-Rs which mediate persistent tonic inhibition, is not ruled out the involvement of this subunit in modulation of chronic pain.[24] Moreover, very interesting is the evidence that the α4 and α5-subunits are expressed in airway smooth muscle (ASM) therefore this $GABA_A$-R subtypes represent a compelling pharmacological target to achieve bronchodilation in asthma and both α4 and α5 $GABA_A$-R positive allosteric modulators could be able to relax precontracted ASM. [25,26]

Continuing our study on $GABA_A$-Rs subtype ligands, in our previous work we synthesized a series of compounds with pyrazolo[1,5-a]quinazoline (PQ)[27] scaffold as 5-deaza analogues of the pyrazolo[5,1-c][1,2,4]benzotriazines (PBT), with anxiolytic, antihyperalgesic[18,28] or procognitive activity,[21] Chart 1. In the new 5-deaza-scaffold, the disubstitution at 3,4 or 3,5 positions, gave compounds lacking of receptorial recognition, and the only compound exhibiting binding affinity is the ethyl pyrazolo[1,5-a]quinazoline 3-carboxylate[27] showing a completely aromatized tricyclic

system. In this work we study the effect of the substitution at 3 and/or 8 position of the PQ scaffold, introducing the same moieties/groups that conferred the best affinity values and selective pharmacological activities to PBT compounds. At the same time, in order to understand to role of ring B of PQ scaffold in the interaction with the target, the synthesis of some 4,5-dihydroderivatives is carried out. The in vitro and in vivo pharmacological assays were then evaluated.

Results and Discussion

Chemistry

All compounds described here are listed in Table S1.

In literature were reported synthetic routes to obtain pyrazolo[1,5-*a*]quinazolines 4,5-dihydro-5-oxo or 5-substituted[29–31], but few papers[32–35] reported the synthesis of 5-unsubstituted. Recently we first reported a multi-step procedure to obtain the pyrazolo[1,5-*a*]quinazoline nucleous 5-unsubstituted[27]. In order to make more efficient and straightforward our previous synthesis two different strategies, following the reported methods [32–35], were planned for the present paper and are reported in Chart 2. The synthetic strategies chosen to obtain the pyrazolo[1,5-*a*]quinazoline scaffold are a copper-catalyzed tandem reaction (route 1) and a Picted-Spengler reaction (route 2).

In the copper-catalyzed tandem reaction, the suitable 2-bromobenzaldehyde of type **1** and the 5-amino-1H-pyrazole-4-carbonitrile **2**, both commercially available, were reacted in high boiling solvent with CuI, K_2CO_3 and NEt_3 as ligand, and afforded the 3-cyanoderivatives **3a, 3b**[32] and **3c** which can be versatile intermediates to obtain desired final compounds (Scheme 1, panel a). Unfortunately, this simple and direct synthetic procedure was not sufficiently convenient overall for the low yield even if diglyme or methoxyethanol as solvent were used. In particular, with methoxyethanol, the alkaline condition of reaction led to a nucleophilic substitution of the group methoxy at the 8-position of the pyrazoloquinazoline with the 2-methoxyethoxy group (compound **3c)**.

As regard the second route (Scheme 1, panel b) the ethyl 1-(3-methoxyphenyl)-5-aminopyrazole 3-carboxylate **4**,[36] was reacted with formaldehyde or paraformaldehyde in acidic medium [33,34] but, the

work up to separate the 8-methoxy isomer **5a** from the 6-methoxy isomer **6** and the unreacted starting material was very difficult.

Thus, the original synthetic procedure was recovered (Scheme 2) and starting from the 2-carboxy-5-methoxyphenylhydrazine (2-hydrazino-4-methoxybenzoic acid) **7**[37], the ethyl 8-methoxy-5-oxo-4,5-dihydropyrazolo[1,5-*a*]quinazoline-3-carboxylate **8** was obtained. The 5-chloroderivative **9**, achieved by reaction with $POCl_3/PCl_5$, was reduced with ammonium formate to ethyl 8-methoxy-4,5-dihydropyrazolo[1,5-*a*]quinazoline 3-carboxylate **10** and, the next oxidation gave the desired aromatic key product, **5a**. The other key compound, the ethyl pyrazolo[1,5-*a*]quinazoline 3-carboxylate[27] is numbered **5b** in this paper. Compounds **5a** and **5b** were hydrolyzed to the corresponding 3-carboxylic acids, **11a, b**[38] that in turn were transformed into the 3-ester derivatives, by treatment with $SOCl_2$ and suitable alcohol (final compounds **12a, b, e** and of type **13**) or with ClCOOEt, NEt_3 and suitable alcohol (final **12c, d**) (Scheme 3).

Finally, compounds **12a, b, d** were transformed into the corresponding 4,5-dihydroderivatives **14a, b, d** by treatment with $NaBH_3CN$ and acetic acid.

In vitro binding

The BZ site/$GABA_A$-R binding affinity of newly synthesized compounds was evaluated by their ability to displace the [^{3}H]flumazenil (Ro15-1788) from its specific binding in bovine brain membrane and was expressed as K_i only for those compounds inhibiting radioligand binding by more than 80% at fixed concentrations of 10 μM.

The binding data (Table 1) show that the almost entirely new compounds have binding recognition in the nanomolar range ($3.16<K_i(nM)<529.3$) raising the sub-nanomolar affinity value of 0.27 nM for compound **12b**.

Looking at the results as a whole, it can be highlighted that the 8-methoxy group is the responsible of the interaction with receptor protein (compare **3a** and **3b**) or improves the binding of the 8-unsubstituted compounds (compare **5a** with **5b**, series **12** with series **13**), even if the type of substituent at position 3 plays a critical role for the binding. It is possible to hypothesize that,

analogously to the 'parent compounds' pyrazolo[5,1-c][1,2,4]benzotriazine (PBT)[39] an interdependence between the substituent at position 3 and the substituents at position 8 exists. Analyzing the results in deep it is noteworthy that, while the 5-oxo-4,5-dihydroderivative **8**, completely lacks of binding recognition, confirming our previous results[27] the corresponding 4,5-dihydroderivative **10**, shows a K_i value of 376 nM. Even though this value is not very high in term of affinity, it is a useful result for studies of molecular modeling and for the development of new compounds.

Within the 3-ester series (ethyl-, benzyl-, 2-methoxybenzyl-, 2-thienylmethyl- 2-furylmethyl- and 2-isopropyl ester), compounds **5a-b**, **12a-e**, and **13a-c**, apart from the importance of the 8-methoxy group to enhance the binding above highlighted, the type of aromatic ring in the ester function is an essential element for the binding affinity. In fact, the ability of the 2-methoxybenzyloxycarbonyl group to engage hydrogen bond interaction with receptor protein through the oxygen atom of methoxy group and the oxygen atom of the carbonyl group, is the explanation for the best affinity value of the compounds **12b** and **13b** (K_i = 0.27 nM and 3.16 nM, respectively). The difference of about an order of magnitude between **12b** and **13b** is exclusively due to the 8-methoxy group that, analogously to PBT compounds[40], engages a hydrogen bond interaction (data not published). On the other hand, the presence of the aryl moiety in the ester function, strongly contributes to the better interaction with lipophilic pocket of receptor protein through WdW interaction, and this can explain the high affinity value of **12b** and **13b** (K_i = 0.27 nM and 3.16 nM, respectively), with respect the 3-alkyl ester **5b, a** and **12e** (K_i = 529.3 nM, 102.4 nM and 97.4 nM, respectively).

As regarding the 4,5-dihydroderivatives, **14a** and **14b** , they have a reduced affinity in comparison with the corresponding aromatic derivatives **12a** and **12b**; while for **14a** the fall of affinity is about two times (**12a**, K_i = 19.0 nM and **14a** K_i = 34 nM), for compound **14b** the difference with **12b** is about 59 times, even if the affinity value remains in the nanomolar range: **12b**, K_i= 0.27 nM and **14b** K_i= 16 nM. The reduced affinity of the dihydroderivatives could be due to the non- planarity of the reduced system due to the presence of $-N^4H-CH_2$. In particular this geometry could influence

the cooperative interaction between the 2-methoxybenzyloxycarbonyl group and the receptor protein.

In vivo testing

The best compound in term of affinity value, **12b**, was further investigated in vivo to assess the potential effects on anxiety, convulsion and amnesia. Then, the antihyperalgesic effect in different pain model was investigated.

The effects on mouse anxiety were studied using a light-dark box apparatus: compound **12b** (10-30 mg/kg po) showed good anxiolytic–like effect with efficacy comparable to diazepam (positive reference) and was completely antagonized by pretreatment with 100 mg/kg flumazenil (a $GABA_AR$ antagonist, used at the same dose able to antagonize the anxiolytic effect of diazepam) suggesting that its anxiolytic-like activity is exerted through the $GABA_A$ subtype receptors (Table 2).

Since the dual anxiolytic-antihyperalgesic action can be therapeutically useful, compound **12b** was also evaluated in a peripheral mono-neuropathy pain model according to the method described by Bennett and Xie[41] at the same dose exerting anxiolytic-like effect (10-30 mg/kg). The nociceptive threshold in the rat was determined with an analgesimeter[42] and 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 (ipsilateral) was observed. By contrast, in the contralateral paw the pain perception remained unchanged. Compound **12b**, showed statistical antihyperalgesic activity only at 30 mg/kg, and the effect was observable 15 min after administration. Moreover, **12b** at the active dose did not modify the pain threshold in the contralateral, non-operated paw, demonstrating the lack of any analgesic effect (data not reported in Table 3).

The hyperalgesia induced by streptozotocine (STZ) and its reversal caused by the treatment with **12b** (30 mg/kg) 15 and 30 min after its administration, is evidenced in the murine hot plate test and after a single injection, (Table 4).

Protection from convulsions was evaluated in mice using pentylentetrazole (PTZ) as chemical convulsant agent. Compounds **12b (**10-100 mg/kg) was devoid of any effect on PTZ-shock whereas diazepam (10 mg/kg), the reference drug, completely protected against PTZ-induced shocks and convulsions, (Table 5). This investigation was necessary since the relevant role of antiepileptic drugs in relieving neuropathic painful conditions.[43–45] This lack of anticonvulsant effect suggests an antihyperalgesic mechanism not identifiable with antiepileptic activity, even if it cannot be completely excluded (pharmacological effects could have on-sets at different concentrations).

To evaluate possible amnesic amnesic effect of **12b**, the passive avoidance test was performed. **12b** (30 mg/kg) did not induce *per se* memory impairment (Table 6), further when amnesia was induced by scopolamine (1.5 mg/kg), **12b** at 10-30 mg/kg did not modify the cognitive properties of the animals (Table 6). Nevertheless, finally, the potential sedative profile of **12b** was studied performing the Rota Rod and the Hole Board tests in mice after a treatment with **12b** (30 mg/kg) in comparison to diazepam (10 mg/kg). As shown in Tables 7 and 8, **12b**-treated animals showed normal motor coordination as well as spontaneous motility and explorative activity. On the contrary, diazepam significantly reduced all these parameters.

Citotoxicity assays

To obtain preliminary data on the toxicity profile of the new compound, human neuronal-like cells were employed.[46,47] These cells were incubated for 72 h with different concentrations of **12b**; at the end of treatments, cell viability was assessed by MTS assay. As shown in the Figure 1, the compound did not significantly affect the viability of neuronal-like cells in a range of concentrations between 1 nM and 1 μM, thus demonstrating its lack of toxicity.

Conclusion

In this paper new 8-methoxypyrazolo[1,5-a]quinazolines as 5-deaza analogues of the pyrazolo[5,1-c][1,2,4]benzotriazines previously reported by us, were synthesized and evaluated for their biological activity on $GABA_A$ subtype receptor. Starting from the lead compound ethyl pyrazolo[1,5-a]quinazoline 3-carboxylate **5b**[27] showing an appreciable affinity value (K_i = 529.3

nM) chemical modification were made either introducing a methoxy group at position 8 or changing the ethoxycarbonyl function into aryl(hetero)alkyl ester moiety, analogously to the reference PBT. The first finding that stands out is that the nitrogen atom at position 5 is not necessary for the binding to receptor protein. Moreover, the pharmacological tests evidenced for the most affine compound **12b** (K_i = 0.27 nM) a profile of positive allosteric modulator (PAM), with anxiolytic and antihyperalgesic activity, lacking of toxicity, when tested in human neuronal-like cells and in vivo models. In fact compound **12b** doesn't modify the motor coordination as well as the spontaneous motility and explorative activity, when tested at 30 mg/kg (the higher dose used) suggesting the lack of neurological and muscular alterations.

The presence of an electron-donor group (MeO) at position 8 of this new PQ other than to enhance the affinity value, seems to be responsible unlike the corresponding PBT[40,48,49], for PAM in vivo profile.

The lack of anticonvulsant- and memory-related-effects, suggested a possible preference of this compound for subunits α2 and α3 (acting as a α2/α3 GABAAR positive allosteric modulator) that could justify the activity on pain threshold modulation. On the contrary subunits α1 and α5 seem to be less involved even if these hypotheses need further investigation to reach an exact knowledge of the pharmacodynamic profile. Nevertheless, it is a matter of fact the interesting characteristics of a new compound active against anxiety and pain in the absence of myorelaxant and amnesic effects.

Very intriguing results are showed by the 4,5-dihydroderivatives, **14a** and **14b**, bearing an ester group at position 3. Despite the good affinity value, K_i = 34 nM and K_i = 16 nM, respectively, it is particularly evident the fall of affinity with respect to the corresponding aromatic derivatives, in particular **14b** versus **12b**. This reduced affinity, could be due to the non-perfect planarity of scaffold caused by the presence of the $-N^4H-CH_2-$ that could influence the cooperative interaction between the 2-methoxybenzyloxycarbonyl group, and the receptor protein.

Studies are in progress to evaluate the size, the steric and stereochemical feature of 8-O-substituent by the synthesis of corresponding 8-alkyloxy/aryloxy/alrylalkyloxyderivatives for the 3-ester derivatives, also in 4,5-dihydroderivatives.

Experimental section

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 400MHz). 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 ≥ 95%: microanalyses were performed with a Perkin-Elmer 260 analyzer for C, H, N.

8-Methoxypyrazolo[1,5-a]quinazoline 3-carbonitrile (3a)

To a solution of 2-bromo-4-methoxybenzaldehyde (1.0 mmol) and 5-aminopyrazolo-4-carbonitrile (1.2 mmol) in dyglime (5 ml) were added CuI (0.2 mmol) and K_2CO_3 (2.0 mmol) and DBU (1,5-diazabiciclo[5.4.0]undec-5-ene, 0.2 mmol). The mixture was refluxed until the starting material disappeared by TLC. It was cooled to room temperature and ice /water was added and the solution was extracted with ethyl acetate. After drying of organic layer with anhydrous Na_2SO_4 and evaporation under vacuum, the final residue was purified by column chromatography (CH_2Cl_2/MeOH 9:1 v/v); pale-yellow crystals, yield 11%; IR ν cm^{-1} 2233; ^{1}H-NMR (DMSO-d_6) δ 9.26 (s, 1H, H-5); 8.76 (s, H-2); 8.26 (d, 1H, H-6, J=8.8 Hz); 7.78 (d, 1H, H-9, J=2.4 Hz); 7.37 (dd, H-7, J=8.8 Hz, J= 2.4 Hz); 4.03 (s, 3H, OCH_3). Anal C, H, N.

8-(2-Methoxyethoxy)pyrazolo[1,5-a]quinazoline 3-carbonitrile (3c)

To a solution of 2-bromo-4-methoxybenzaldehyde (1.0 mmol) and 5-aminopyrazolo-4-carbonitrile (1.2 mmol) in 2-methoxyethanol (5 ml) were added CuI (0.2 mmol) and K_2CO_3 (2.0 mmol) and DBU (1,5-diazabiciclo[5.4.0]undec-5-ene, 0.2 mmol). The mixture was refluxed until the starting

material disappeared by TLC. It was cooled to room temperature and ice /water was added and the solution was extracted with ethyl acetate. After drying of organic layer with anhydrous Na_2SO_4 and evaporation under vacuum, the final residue was purified by column chromatography (CH_2Cl_2/MeOH 9:1 v/v); pale-yellow crystals, yield 25%; IR ν cm^{-1} 2243; ^{1}H-NMR (DMSO-d_6) δ 9.26 (s, 1H, H-5); 8.77 (s, H-2); 8.26 (d, 1H, H-6, J=8.8 Hz); 7.80 (d, 1H, H-9, J=2.4 Hz); 7.38 (dd, H-7, J=8.8 Hz, J= 2.4 Hz); 4.40 (t, 2H, CH_2O, J=4.0 Hz); 3.73 (t, 2H, CH_3OCH_2, J=4.0 Hz); 3.28 (s, 3H, CH_3O). Anal C, H, N.

Synthesis of compounds 5a and 6 *via* Pictet-Spengler approach.

A solution of ethyl 1-(3-methoxyphenyl)-5-aminopyrazole 3-carboxylate[36] **4**, (1.0 mmol) in 6M HCl (5 ml), was added of excess of paraformaldehyde and refluxed evaluating the course of reaction by TLC (toluene/ethyl acetate /methanol 8:2:1.5 v/v/v). The solution was evaporated to dryness and the two isomers, **5a** and **6**, were separated by chromatography column with the same eluent.

Compound **5a** was also obtained via a standard synthetic approach. An excess of Pd/C (10%, 100 mg) was added to a solution of **10** (0.350 mmol) in 5 ml of toluene (the synthesis of compound **10** was reported below). The mixture was refluxed for 1 hour followed by the filtration of catalyst and the next evaporation of the solvent. A yellow residue, recovered by i-propyl ether, was obtained.

Ethyl 8-methoxypyrazolo[1,5-a]quinazoline-3-carboxylate (5a)

From Pictet-Spengler approach, yield: 15%; from standard synthetic approach yield: 55%. IR ν cm^{-1} 1681; ^{1}H-NMR (DMSO-d_6): δ 9.22 (s, 1H, H-5); 8.52 (s, 1H, H-2), 8.21 (d, 1H, H-6, J=8.8 Hz), 7.79 (d, 1H, H-9, J=2.4 Hz), 7.32 (dd, 1H, H-7, J=8.8 Hz, J=2.4 Hz), 4.30 (q, 2H, CH_2, J=7.2 Hz); 4.02 (s, 3H, OCH_3); 1.31 (t, 3H, CH_3, J=7.2 Hz). Anal C, H, N.

Ethyl 6-methoxypyrazolo[1,5-a]quinazoline-3-carboxylate (6)

Yield: 18%. IR ν cm^{-1} 1683; ^{1}H-NMR (DMSO-d_6): δ 9.42 (s, 1H, H-5); 8.57 (s, 1H, H-2), 8.02 (t, 1H, H-8, J=8.0 Hz), 7.95 (d, 1H, H-9, J=8.0 Hz), 7.26 (d, 1H, H-7, J=8.0 Hz), 4.30 (q, 2H, CH_2, J=7.2 Hz); 4.05 (s, 3H, OCH_3); 1.31 (t, 3H, CH_3, J=7.2 Hz). Anal C, H, N.

Ethyl 8-methoxy-5-oxo-4,5-dihydropyrazolo[1,5-a]quinazoline 3-carboxylate (8)

To a suspension of 2-hydrazino-4-methoxybenzoic acid (2.42 mmol) in DMF (10 ml) was added sodium acetate (2.9 mmol) and ethyl 2-cyano-3-ethoxyacrilate (2.42 mmol). The reaction was maintained at reflux temperature until the starting material disappeared, evaluated by TLC (toluene/ethyl acetate /acetic acid 8:2:1 v/v/v). The final solution was treated with ice/water and crude product precipitate; it was filtered and recrystallized by suitable solvent. Beige crystals, yield 85%; IR ν cm^{-1} 3425, 1709, 1679; ^{1}H-NMR (DMSO-d_6) δ 11.39 (bs, 1H, NH, exch.); 8.18 (s, 1H, H-2); 8.10 (d, 1H, H-6, J=8.8 Hz); 7.51 (d, 1H, H-9, J=2.4 Hz); 7.14 (dd, H-7, J=8.8 Hz, J= 2.4 Hz); 4.31 (q, 2H, CH_2, J=7.2 Hz); 3.97 (s, 3H, OCH_3); 1.31 (t, 3H, CH_3 J=7.2 Hz). Anal C, H, N.

Ethyl 8-methoxy-5-chloropyrazolo[1,5-a]quinazoline 3-carboxylate (9)

To a suspension of compound **8** (0.35 mmol) in $POCl_3$ (5 ml) was added PCl_5 (0.36 mmol) and was maintained at reflux temperature. At the end of reaction a yellow solution was obtained and it was evaporated to dryness; ice /water was added to precipitate the final light brown product, which was enough pure to be used in the next reaction. Straw-yellow crystals, yield 70%; IR ν cm^{-1} 1716; ^{1}H-NMR (DMSO-d_6) δ 8.51 (s, 1H, H-2); 8.22 (d, 1H, H-6, J=9.2 Hz); 7.88 (d, 1H, H-9, J=2.4 Hz); 7.23 (dd, H-7, J=9.2 Hz, J= 2.4 Hz); 4.47 (q, 2H, CH_2, J=7.2 Hz); 4.01 (s, 3H, OCH_3); 1.46 (t, 3H, CH_3 J=7.2 Hz). Anal C, H, N.

Ethyl 8-methoxy-4,5-dihydropyrazolo[1,5-a]quinazoline 3-carboxylate (10)

To a suspension of **9** (0.300 mmol) in 3.5 ml of THF anhydrous was added 25 mg of Pd/C 10%, 1.0 mmol of ammonium formate and 10 ml of methanol. The reaction was maintained at reflux temperature, monitored by TLC (toluene/ethyl acetate/ methanol 8:2:1.5 v/v as eluent) and after 2.5 hours the starting material was disappeared. The filtration of catalyst and the next evaporation of the solution gave a residue that was recovered by *i*-propyl ether. Off-white crystals; Yield: 72%. IR ν cm^{-1} 3386, 1666; ^{1}H-NMR ($CDCl_3$): δ 7.71 (d, 1H, H-2), 7.26 (d, 1H, H-9), 7.01 (d, 1H, H-6, J=8.4 Hz), 6.69 (dd, 1H, H-7, J=8.4 Hz, J=2.8), 5.84 (bs, 1H, NH exchange), 4.58 (s, 2H, CH_2NH); 4.27 (q, 2H, CH_2, J=7.2 Hz); 3.84 (s, 3H, OCH_3); 1.26 (t, 3H, CH_3, J=7.2 Hz). Anal C, H, N.

General procedure for the synthesis of compounds 11a and 11b.

A suspension of compounds **5a** or **5b**[27] (0.18 mmol) in a 10% sodium hydroxide solution (6 ml) was refluxed for 3h. The final solution was cooled and acidified with HCl conc.; the precipitate was filtered, washed with water and purified by suitable solvent.

8-Methoxypyrazolo[1,5-a]quinazoline-3-carboxylic acid (11a)

From ester **5a**. White crystals by ethanol; Yield: 90%. IR ν cm^{-1} 3513-3200, 1687; ^{1}H-NMR (DMSO-d_6): δ 12.5 (bs, 1H, OH); 9.18 (s, 1H, H-5); 8.50 (s, 1H, H-2), 8.20 (d, 1H, H-6, J= 8.8 Hz); 7.77 (d, 1H, H-9, J=2.0 Hz); 7.31 (dd, 1H, H-7, J=8.8 Hz, J=2.0 Hz); 4.02 (s, 3H, OCH_3). Anal C, H, N.

Pyrazolo[1,5-a]quinazoline-3-carboxylic acid (11b)

From ester **5b.**[27] White crystals from ethanol; Yield: 61%. IR ν cm^{-1} 3300-2800, 1665; ^{1}H-NMR (DMSO-d_6): δ 12.0 (bs, 1H, OH); 9.34 (s, 1H, H-5); 8.54 (s, 1H, H-2), 8.43 (d, 1H, H-9, J= 8.4 Hz); 8.29 (d, 1H, H-6, J=8.4 Hz), 8.09 (t, 1H, H-8, J=8.4); 7.74 (t, 1H, H-7, J=8.4 Hz). Anal C, H, N.

General procedure for the synthesis of compounds 12a-12e and 13a-13c.

The starting acid **11a** and **11b** were transformed into ester derivatives (**12a-12e** and **13a-13c**) by means two method A and B.

Method A. The esters **12a, 12b, 12e** and **13a-13c** were obtained from the acids **11a** and **11b** (0.5 mmol) respectively, by means of synthesis of the corresponding 3-carbonyl chloride, which was in turn suspended in dichloromethane and added of the suitable alcohol. The final solution was evaporated to dryness and the residue recuperated with isopropyl ether and recrystallized.

Method B. The esters **12c** and **12d** were obtained by treating the acid **11a** (0.5 mmol) in tetrahydrofurane (THF), with triethyl amine (1:3.5) in ice bath for 30 min. The suspension was supplemented with ethyl chlorocarbonate (1:2) and maintained from 0 °C to room temperature under stirring for 1 h to permit the anhydride to form. Alcohol was added (1:2.5) and the mixture was heated at 60 °C for 6-8 h and monitored by TLC. The final suspension was diluted with water and extracted with dichloromethane that was, in turn, washed with sodium hydrogen carbonate

solution and, after normal work up the residue was treated with isopropyl ether or ethyl ether and recrystallized by suitable solvent.

Benzyl 8-methoxypyrazolo[1,5-a]quinazoline-3-carboxylate (12a)

From **11a** and benzyl alcohol, (method A). White crystals from ethanol; Yield: 48%. IR ν cm^{-1} 1712; ^{1}H-NMR (DMSO-d_6): δ 9.23 (s, 1H, H-5); 8.61 (s, 1H, H-2), 8.20 (d, 1H, H-6, J= 8.8 Hz); 7.78 (s, 1H, H-9), 7.49 (m, 2H, H-7 and benzyl); 7.38 (m, 2H, benzyl); 7.32 (d, 2H, benzyl, J=8.0 Hz); 5.35 (s, 2H, CH_2); 4.01 (s, 3H, OCH_3). ^{13}C-NMR (DMSO-d_6): 165.3, 155.2, 145.6, 137.7, 131.7, 129.7, 129.0, 124.8, 120.8, 117.5, 113.8, 111.3, 96.4, 61.1, 56.8. Anal C, H, N.

2-Methoxybenzyl 8-methoxypyrazolo[1,5-a]quinazoline-3-carboxylate (12b)

From **11a** and 2-methoxybenzyl alcohol, (method A). White crystals from ethanol; Yield: 62%. IR ν cm^{-1} 1710; ^{1}H-NMR (DMSO-d_6): δ 9.24 (s, 1H, H-5); 8.61 (s, 1H, H-2); 8.22 (d, 1H, H-6, J=8.8 Hz); 7.79 (s, 1H, H-9); 7.51 (d, 1H, H-7, J=8.4 Hz), 7.32 (m, 2H, H-6 and H-4 benzyl); 7.03 (d, 1H, H-3 benzyl, J=8.4 Hz); 6.96 (t, 1H, H-5 benzyl, J=7.2 Hz); 5.32 (s, 2H, CH_2); 4.02 (s, 3H, OCH3); 3.81 (s, 3H, OCH_3). ^{13}C-NMR (DMSO-d_6): 165.3, 155.2, 145.6, 137.7, 131.7, 129.7, 129.5, 129.0, 124.8, 120.8, 117.5, 113.8, 111.3, 96.4, 61.1, 56.8, 55.9. Anal C, H, N.

Thiophen-2-ylmethyl 8-methoxypyrazolo[1,5-a]quinazoline-3-carboxylate (12c)

From **11a** and 2-thiophenmethanol, (method B). White crystals from chromatography column, toluene/ethyl acetate/methanol 8:2:1.5 v/v/v; Yield: 15%. IR ν cm^{-1} 1715; ^{1}H-NMR (DMSO-d_6): δ 9.24 (s, 1H, H-5); 8.59 (s, 1H, H-2); 8.22 (d, 1H, H-6, J=8.8 Hz); 7.79 (d, 1H, H-9, J= 2.4 Hz); 7.54 (d, 1H, H-5 thiophene, J=5.2 Hz); 7.33 (dd, 1H, H-7, J=8.8 Hz, J=2.4 Hz); 7.25 (d, 1H, H-3 thiophene, J=3.2 Hz); 7.02 (m, 1H, H-4 thiophene); 5.50 (s, 2H, CH_2); 4.02 (s, 3H, OCH3). ^{13}C-NMR (DMSO-d_6): 165.3, 161.8, 155.1, 145.6, 145.4, 131.7, 117.4, 113.5, 111.0, 96.4, 57.9, 56.8. Anal C, H, N.

Furan-2-ylmethyl 8-methoxypyrazolo[1,5-a]quinazoline-3-carboxylate (12d)

From **11a** and 2-furanmethanol, (method B). White crystals from chromatography column, dichloromethano/methanol 8:2 v/v; Yield: 28%. IR ν cm^{-1} 1715; ^{1}H-NMR (DMSO-d_6): δ 9.23 (s,

1H, H-5); 8.57 (s, 1H, H-2); 8.21 (d, 1H, H-6, J=8.8 Hz); 7.79 (d, 1H, H-9, J= 2.0 Hz); 7.69 (s, 1H, H-5 furane); 7.33 (dd, 1H, H-7, J=8.8 Hz, J=2.0 Hz); 6.58 (d, 1H, H-3 furane, J=3.2 Hz); 6.47 (m, 1H, H-4 furane); 5.30 (s, 2H, CH_2); 4.02 (s, 3H, OCH3). ^{13}C-NMR (DMSO-d_6): 165.3, 161.8, 155.2, 150.2, 145.6, 145.3, 137.7, 131.8, 117.2, 113.8, 111.2, 103.9, 96.3, 57.6, 56.8, 55.9. Anal C, H, N.

Propan-2-yl 8-methoxypyrazolo[1,5-a]quinazoline-3-carboxylate (12e)

From **11a** and 2-propanol, (method A). White crystals from ethanol; Yield: 48%. IR ν cm^{-1} 1707; ^{1}H-NMR (DMSO-d_6): δ 9.22 (s, 1H, H-5); 8.53 (s, 1H, H-2); 8.20 (d, 1H, H-6, J=8.8 Hz); 7.79 (s, 1H, H-9); 7.32 (d, 1H, H-7, J=8.4 Hz), 5.14 (m, 1H, CH); 4.02 (s, 3H, OCH3); 1.31 (d, 6H, $(CH_3)_2$, J=6.4). ^{13}C-NMR (DMSO-d_6): 165.3, 162.3, 155.0, 145.6, 145.2, 137.7, 131.8, 128.3, 117.5, 113.8, 104.6, 96.3, 67.3, 56.8, 21.6. Anal C, H, N.

Benzyl pyrazolo[1,5-a]quinazoline-3-carboxylate (13a)

From **11b** and benzyl alcohol, (method A). White crystals from ethanol; Yield: 25%. IR ν cm^{-1} 1712; ^{1}H-NMR ($CDCl_3$): δ 9.22 (s, 1H, H-5); 8.53 (m, 2H, H-2 and H-6), 8.05 (d, 1H, H-9, J= 8.4 Hz); 7.99 (t, 1H, H-7, J=8.4 Hz), 7.60 (t, 1H, H-8, J=8.4); 7.53 (m, 2H, benzyl); 7.38-7.32 (m, 3H, benzyl); 5.45 (s, 2H, CH_2). ^{13}C-NMR (DMSO-d_6): 165.3, 155.2, 145.6, 137.7, 131.7, 129.7, 129.0, 124.8, 120.8, 117.5, 113.8, 111.3, 96.4, 61.1. Anal C, H, N.

2-Methoxybenzyl pyrazolo[1,5-a]quinazoline-3-carboxylate (13b)

From **11b** and 2-methoxybenzyl alcohol, (method A). White crystals from ethanol; Yield: 53%. IR ν cm^{-1} 1715; ^{1}H-NMR (DMSO-d_6): δ 9.40 (s, 1H, H-5); 8.63 (s, 1H, H-2); 8.45(d, 1H, H-6, J=8.4 Hz); 8.30 (d, 1H, H-9, J= 8.4 Hz); 8.10 (t, 1H, H-7, J=8.4 Hz), 7.76 (t, 1H, H-8, J=8.4); 7.51 (d, 1H, H-6 benzyl, J=7.2 Hz); 7.32 (t, 1H, H-5 benzyl, J=7.2 Hz); 7.03 (d, 1H, H-3 benzyl, J=7.2 Hz); 6.96 (t, 1H, H-4 benzyl, J=7.2 Hz); 5.33 (s, 2H, CH_2); 3.81 (s, 3H, OCH_3). ^{13}C-NMR (DMSO-d_6): 165.3, 155.2, 145.6, 137.7, 131.7, 129.7, 129.5, 129.0, 124.8, 120.8, 117.5, 113.8, 111.3, 96.4, 61.1, 55.9. Anal C, H, N.

Thiophen-2-ylmethyl pyrazolo[1,5-a]quinazoline-3-carboxylate (13c)

From **11b** and 2-thiophenmethanol, (method A). White crystals from ethanol; Yield: 55%. IR ν cm^{-1} 1715; ^{1}H-NMR (DMSO-d_6): δ 9.39 (s, 1H, H-5); 8.60 (s, 1H, H-2); 8.44 (d, 1H, H-6, J=8.4 Hz); 8.30 (d, 1H, H-9, J= 8.4 Hz); 8.10 (t, 1H, H-7, J=8.4 Hz), 7.76 (t, 1H, H-8, J=8.4); 7.54 (d, 1H, H-5 thiophene, J=5.2 Hz); 7.25 (d, 1H, H-3 thiophene, J=3.2 Hz); 7.02 (m, 1H, H-4 thiophene); 5.52 (s, 2H, CH_2). ^{13}C-NMR (DMSO-d_6): 165.3, 161.8, 155.1, 145.6, 145.4, 131.7, 117.4, 113.5, 111.0, 96.4, 57.9. Anal C, H, N.

General procedure for the synthesis of compounds 14a, 14b and 14d.

The starting ester **12a**, **12b** and **12d** (0.240 mmol) were solubilized in glacial acetic acid, under nitrogen flow. Then, 0.972 mmol of sodium cyanoborohydride ($NaBH_3CN$) was added, the reaction was maintained at 50 °C for 3 h and monitored by TLC. The final solution was cooled at room temperature and water added until a precipitate was formed; the raw product was purified by chromatography column or by recrystallization by suitable solvent.

Benzyl 8-methoxy-4,5-dihydropyrazolo[1,5-a]quinazoline-3-carboxylate (14a)

From **12a**. White crystals by chromatography column (eluent toluene/ethyl acetate 9:1 v/v); Yield: 37%. IR ν cm^{-1} 3396, 1712, 1246, 1108; ^{1}H-NMR (DMSO-d_6): δ 7.72 (s, 1H, H-2); 7.40-7.29 (m, 5H, Ph), 7.23 (bs, 1H, NH, exch.); 7.19 (d, 1H, H-6, J=8.0 Hz); 7.06 (d, 1H, H-9, J=2.4 Hz); 6.73 (dd, 1H, H-7, J=8.0 Hz, J=2.4 Hz); 5.22 (s, 2H, CH_2); 4.46 (s, 2H, CH_2NH); 3.75 (s, 3H, OCH_3). ^{13}C-NMR (DMSO-d_6): 162.7, 159.8, 148.9, 141.8, 137.4, 135.0, 128.8, 128.2, 128.2, 112.5, 111.72, 99.7, 94.1, 64.7, 55.8, 42.2. Anal C, H, N.

2-Methoxybenzyl 8-methoxy-4,5-dihydropyrazolo[1,5-a]quinazoline-3-carboxylate (14b)

From **12b**. White crystals from *i*-propanol; Yield: 50%. IR ν cm^{-1} 3392, 1710, 1246, 1108; ^{1}H-NMR (DMSO-d_6): δ 7.71 (s, 1H, H-2); 7.29 (m, 2H, H-6 and benzyl); 7.20 (bs, 1H, NH exch.); 7.17 (s, 1H, benzyl); 7.06 (d, 1H, H-9, J=2.0 Hz); 7.01 (d, 1H, benzyl, J=8.4 Hz); 6.92 (t, 1H, benzyl, J=7.2); 6.73 (dd, 1H, H-7, J=8.4 Hz, J=2.0 Hz); 5.25 (s, 2H, CH_2); 4.46 (s, 2H, CH_2NH); 3.80 (s, 3H, OCH3); 3.75 (s, 3H, OCH_3). ^{13}C-NMR (DMSO-d_6): 162.8, 157.3, 148.4, 141.6, 134.7, 129.7, 129.5, 129.0, 120.7, 111.3, 108.6, 108.3, 106.5, 93.5, 60.3, 56.2, 55.9, 38.4. Anal C, H, N.

Furan-2-ylmethyl 8-methoxy-4,5-dihydropyrazolo[1,5-a]quinazoline-3-carboxylate (14d)

From **12d**. White crystals from *i*-propanol; Yield: 75%. IR ν cm^{-1} 3392, 1715, 1246, 1108; ^{1}H-NMR (DMSO-d_6): δ 7.66 (m, 2H, H-2 and H-6); 7.19 (m, 2H, H-9 and NH, exch.); 7.06 (s, 1H, H-5 furane); 6.73 (dd, 1H, H-7, J=8.8 Hz, J=2.0 Hz); 6.51 (d, 1H, H-3 furane, J=3.2 Hz); 6.48 (m, 1H, H-4 furane); 5.17 (s, 2H, CH_2); 4.45 (4.02 s, 2H, CH_2NH); 3.75 (s, 3H, OCH3). ^{13}C-NMR (DMSO-d_6): 165.4, 161.8, 155.2, 150.2, 145.6, 145.3, 137.7, 131.9, 117.2, 113.8, 111.2, 103.9, 96.3, 57.6, 56.8. 42.3. Anal C, H, N.

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.[50,51] 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.[52] [^{3}H]Ro 15-1788 binding studies were performed as previously reported.[53] 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}, Ki, and SEM values for tested compounds, the Ki values being calculated from the Cheng and Prusoff equation.[54]

Cytotoxicity assay

Cell culture and neuronal differentiation

H9-derived Neural stem cells (NSCs) were plated on polyornithine and laminin-coated culture dishes. In order to achieve a neuronal phenotype, and switched into a defined Neurobasal serum-free medium,[46] containing 2% B-27, 2mM L-glutamine and 5 μM retinoic acid[55] for seven days.

Viability assay

Neuronal-like cells, differentiated from H9-derived NSCs, were incubated for 72 h with 12b (1 nM-1 μM) treated as described above. Following incubation time, cell viability was determined using

the MTS assay.[46] The absorbance was read at 490 nM using an automated plate reader. The mean background from the values was subtracted from each test condition, and the data were expressed as the percentage of the control (untreated cells).

Pharmacological Methods

Animals: Male Swiss albino mice (23-30 g) and male Sprague Dawley rats (200-225 g) from Envigo (Varese, Italy) 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 as well as ARRIVE guidelines.

Drugs: Diazepam (Valium 10 – Roche) and the new synthesized compounds were suspended in 1% carboxymethylcellulose sodium salt, sonicated immediately before use and administered by oral route (p.o.). The other compounds were dissolved in isotonic (NaCl 0.9%) saline solution and injected subcutaneously (s.c.) (PTZ; Sigma) or intraperitoneally (i.p.) (flumazenil and scopolamine; Sigma). Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml/kg by the p.o., i.p. or s.c. routes.

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 x 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.

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.[41] 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 1mm 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.

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.[42] 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.

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,[56] 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 15, 30, 45 and 60 min after the last injection of test compounds.

Hot plate test: The method adopted was described by O'Callaghan and Holzman.[57] 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.

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.

Passive avoidance test: The test was performed according to the step-through method described by Jarvik and Kopp[58] with modifications. The apparatus consisted of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. In the original method mice received a punishing electrical shock as soon as they entered the dark compartment, while in our modified method, mice, after their entry into the dark compartment, received a punishment consisting of a fall into a cold water bath (10 °C). For this purpose the dark chamber was constructed with a pitfall door. The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test. The maximum entry latency allowed in the retention session was 120 s. **12b** was injected 30 min before the training session; the amnesic drug scopolamine was injected immediately after termination of the training session.

Rota rod test: The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus up to 5 mice were tested simultaneously on the apparatus, with a rod rotation speed of 16 r.p.m. The integrity of motor coordination was

assessed on the basis of the number of falls from the rod in 30 s. Measures were performed before and after drugs administration at 15 min intervals.

Hole board test: The locomotor activity was evaluated using the hole-board test. The apparatus consisted of 40 a cm square plane with 6 flush mounted cylindrical holes (3 cm diameter) distributed 4 × 4 in an equidistant, grid like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 5 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signaled the movement of the animals (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

Statistical analysis: All experimental results are given as the mean ± S.E.M. Analysis of variance (One-way 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. Investigators were blind to all experimental procedures.

Supporting Information

Combustion analysis data of the newly synthesized compounds.

Molecular Formula Strings.

Corresponding Author

Phone +39 055 4573766. E-mail: gabriella.guerrini@unifi.it

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Abbreviation used

PBT, pyrazolobenxotriazine; PQ, pyrazoloquinoline; NAM, negative allosteric modulator; PAM, positive allosteric modulator;

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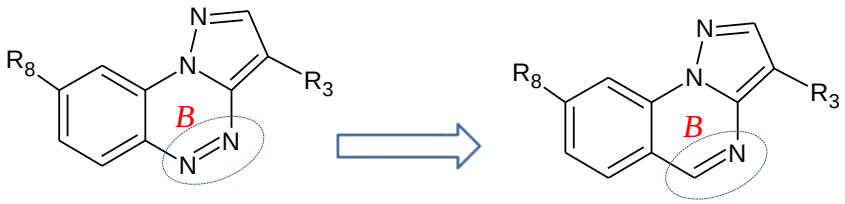
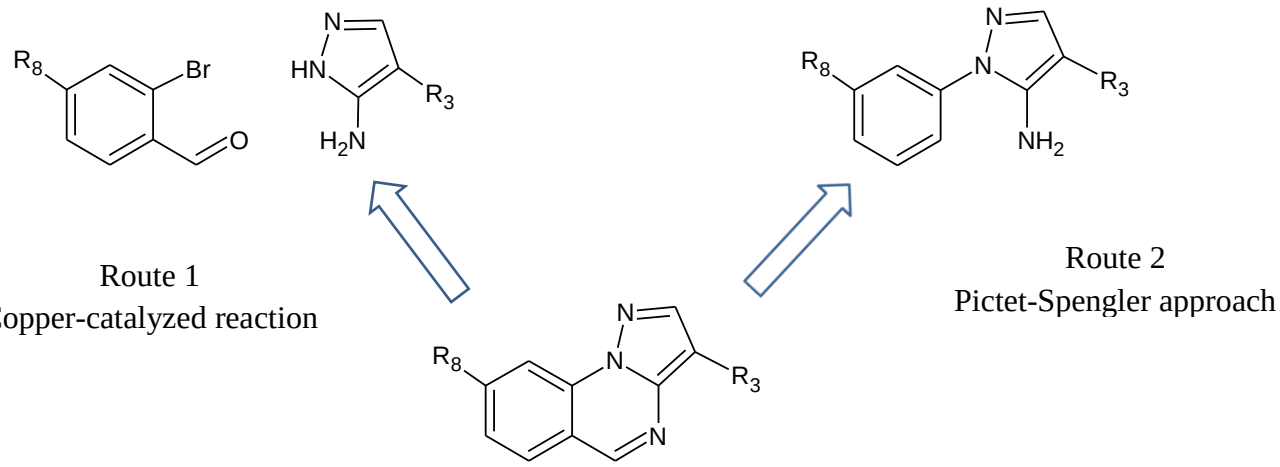
Chart 1



PBT PQ

Chart 2

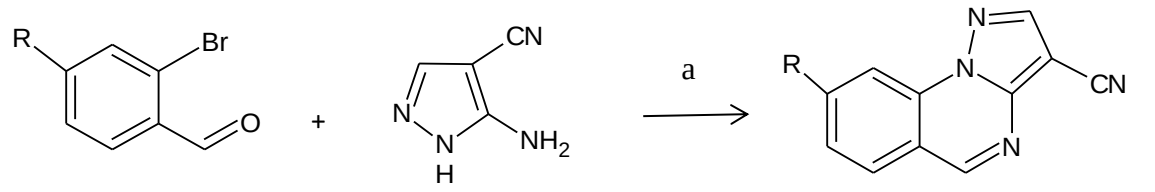


Route 1
Copper-catalyzed reaction

Route 2
Pictet-Spengler approach

Scheme 1

a)



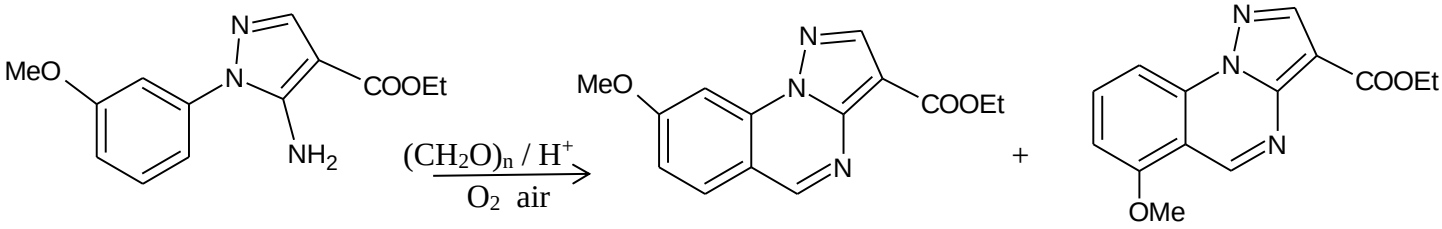
1a, 1b
R = OCH_3, H

2

3a, 3b[32], **3c**
R = OCH_3, H, OCH_2CH_2OMe

Reagents and conditions: a) CuI, K_2CO_3, NEt_3, diglyme for **3a** and **3b**, methoxyethanol for **3c.**

b) Pictet-Spengler approach [33,34]

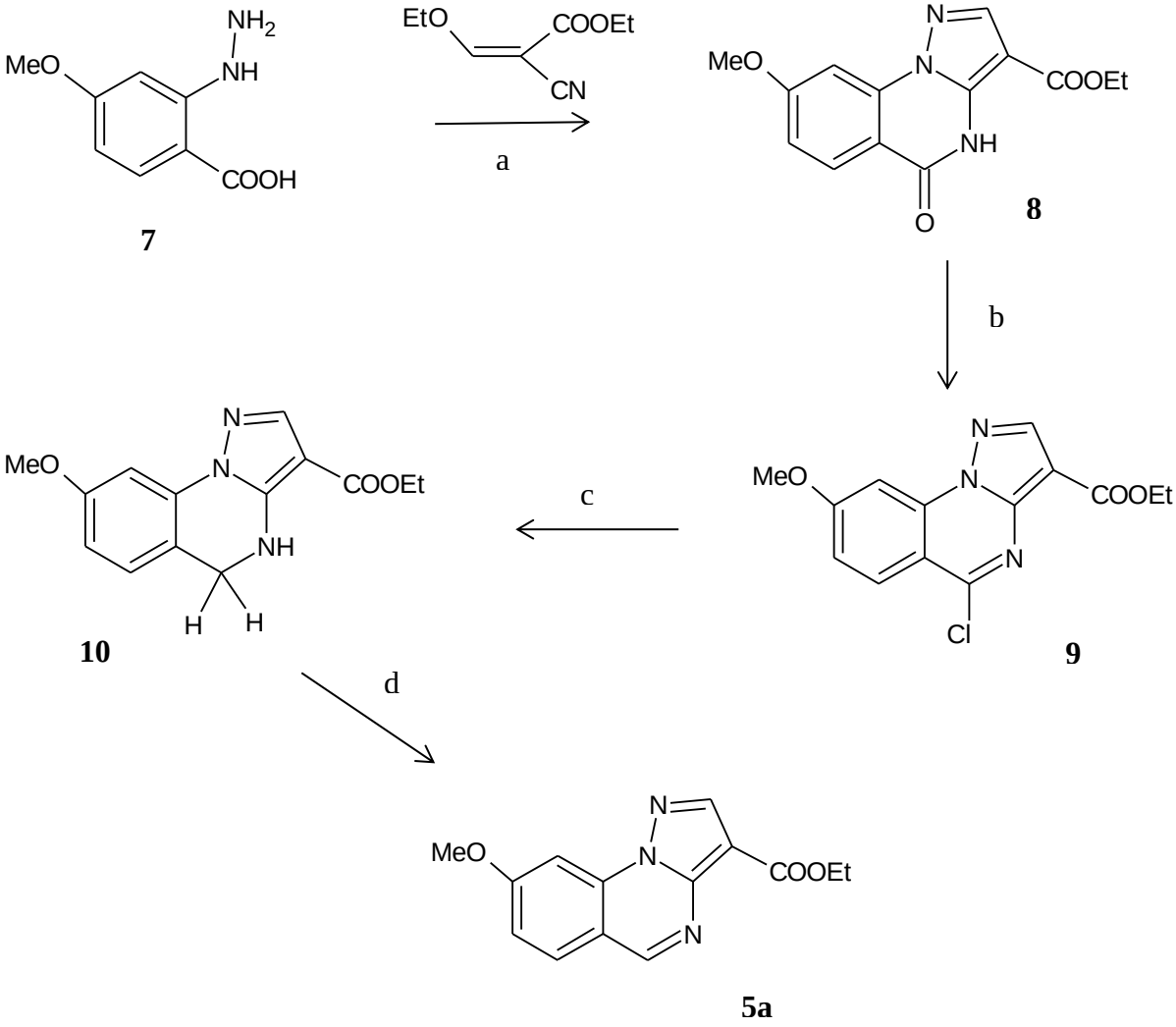


4

5a

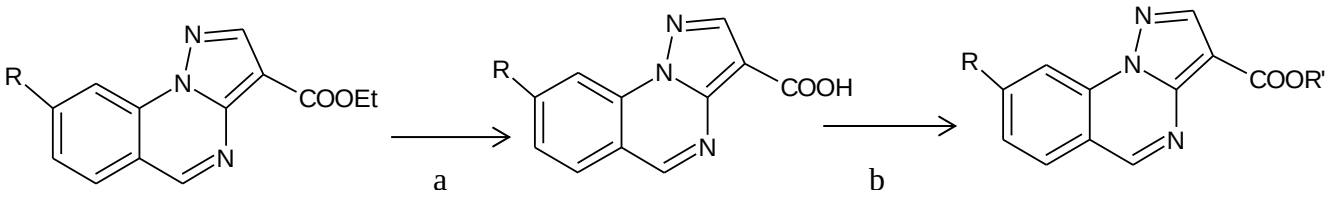
6

Scheme 2



Reagents and conditions: a) DMF/AcONa; b) $POCl_3/PCl_5$; c) EtOH, Pd/C 10%, $HCOONH_4$; d) toluene, Pd/C 10% reflux.

Scheme 3



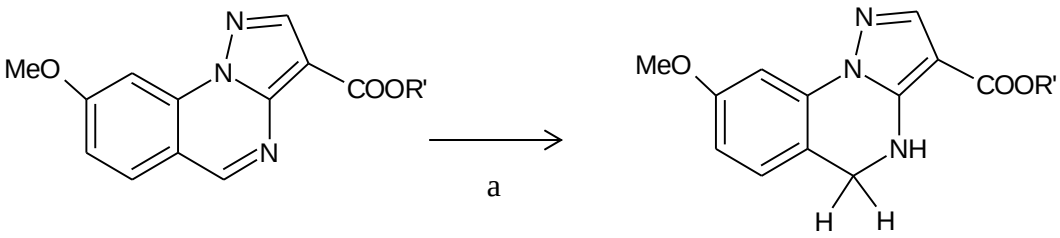
Comp	R
5a	OMe
5b	H[27]

Comp	R
11a	OMe
11b	H

Comp	R	R'
12a	OMe	CH_2Ph
12b	OMe	CH_2-(2-OMe)-Ph
12c	OMe	CH_2-2-thienyl
12d	OMe	CH_2-2-furyl
12e	OMe	CH-$(CH_3)_2$
13a	H	CH_2Ph
13b	H	CH_2-(2-OMe)-Ph
13c	H	CH_2-2-thienyl

Reagents and conditions: a) NaOH 10% reflux, HCl; b) $SOCl_2$ reflux, CH_2Cl_2, $ArCH_2OH$ for **12a**, **12b**,**12e 13a**, **13b**, **13c**; THF abs, NEt_3, 0 °C, ClCOOEt, $ArCH_2OH$ for **12c** and **12d**;

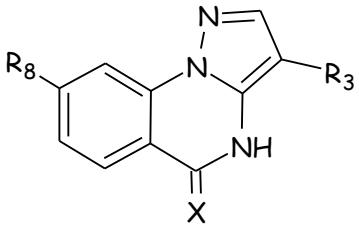
Scheme 4



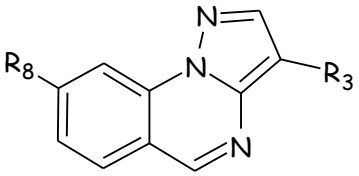
Comp	R’
12a	CH_2Ph
12b	CH_2-(2-OMe)-Ph
12d	CH_2-2-furyl

Comp	R’
14a	CH_2Ph
14b	CH_2-(2-OMe)-Ph
14d	CH_2-2-furyl

Reagents and conditions: a) AcOH, $NaBH_3CN$, 50 °C.



8, **10, 14a-14c**



3a-3c, **5a-5b**, **12a-12c, 13a-13e**

Table 1. BZR ligand affinity of new pyrazolo[1,5-a]quinazoline derivatives

	R_3	R_8	X	I% or K_i (nM) [a]
3a	CN	OMe		378±54
3b	CN	H		27%
3c	CN	OCH_2CH_2OMe		53%
5a	COOEt	OMe		102.4±10.4
5b	COOEt	H		529.3±58.8[b]
8	COOEt	OMe	O	2%
10	COOEt	OMe	H, H	376±54
12a	$COOCH_2Ph$	OMe		19.0±1.9
12b	$COOCH_2$-2-OMe-Ph	OMe		**0.27±0.04**
12c	$COOCH_2$-2-tienyl	OMe		16.7±0.9
12d	$COOCH_2$-2-furyl	OMe		15.2±1.4
12e	$COOCHMe_2$	OMe		97.4±13.8
13a	$COOCH_2Ph$	H		25.9±1.98
13b	$COOCH_2$-2-OMe-Ph	H		**3.16±0.54**
13c	$COOCH_2$-2-tienyl	H		13.2±0.7
14a	$COOCH_2Ph$	OMe	H, H	34±1.3
14b	$COOCH_2$-2-OMe-Ph	OMe	H, H	16±1.0
14d	$COOCH_2$-2-furyl	OMe	H, H	NT
	Diazepam			12.3±1.5

[a] Percent of inhibition of specific [^{3}H]flumazenil (Ro15-1788) binding at 10μM concentration; K_i value are means ±SEM of five determinations. [b] New binding evaluation with respect to reference [27] in which K_i value was 834.7±61.1

Table 2. Effect of **12b** in comparison with DAZ in light-dark box test

Treatment	mg/kg	Persistent time in light box (s)
Vehicle		131.9±6.5
Flumazenil	100 mg/kg	127.5±7.7
Diazepam	10 mg/kg	165.6 ± 9.1*
12b	1 mg/kg	133.1±8.4
12b	10 mg/kg	169.6±6.9*
12b	30 mg/kg	165.7±5.7*
12b + Flumazenil	10 mg/kg + 100 mg/kg	136.4±8.0[§]

Compound **12b** and diazepam were administered p.o. 30 min before the test; flumazenil was administered i.p. 40 min before the test. Control animals were treated with 1% carboxymethylcellulose. Each value represents at least 7-9 mice. *P< 0.01 vs vehicle-treated mice. [§]P<0.01 vs flumazenil-treated mice.

Table 3. Effect of **12b** in a rat model of mononeuropathy evaluated in the paw-pressure test

Treatment mg/kg p.o.		paw	Paw pressure in rats (g)			
			Before treatment	After treatment		
				15 min	*30 min*	*45 min*
Vehicle		contralateral	60.5 ± 3.1	59.1±3.6	56.9±4.3	61.3±4.6
Vehicle		ipsilateral	31.5±4.0	33.7±3.2	34.7±2.5	35.2±3.6
12b	10	ipsilateral	31.3±3.4	39.8±3.0	41.7±3.6	36.9±3.8
	30	ipsilateral	30.3±3.2	52.4±3.6*	41.7±3.6	35.9±3.9

There were at least 5 rats per group. Controls were treated with vehicle (1% carboxymethylcellulose). *P< 0.01 in comparison to measures performed on the ipsilateral paw of vehicle-treated animals.

Table 4. Effect of **12b** on the hyperalgesia induced by streptozotocine in mouse hot-plate test

Pre-treatment	*Licking latency in mice (s)*					
	Treatment mg/kg p.o.	*Before treatment*	*after treatment*			
			15 min	*30 min*	*45 min*	*60 min*
Vehicle	**Vehicle**	14.8±0.8	18.3±1.1	18.8±1.2	18.0±0.9	17.9±1.1
STZ	**Vehicle**	9.7 ± 0.4	9.4±1.1	10.2 ± 0.8	10.9±0.8	9.6±0.9
Vehicle	**12b** 30	16.5±0.8	17.3±1.1	17.2±1.2	18.4±0.9	17.5±1.0
STZ	**12b** 30	10.2±0.7	16.6±1.0*	15.5±0.8*	13.1±1.0	11.2±1.0

Streptozotocine (STZ) 200 mg kg^{-1} i.p. was dissolved in citrate buffer and injected 21 days before experiment. Controls were treated with vehicles. Each value represents the mean of at least 10 mice. * $P< 0.01$ in comparison with STZ-treated mice.

Table 5. Effect of **12b** in PTZ treated mice test

Treatment	*Dose mg/kg p.o.*	*% protection on PTZ induced convulsions*
12b	10	0
	30	10
	100	10
Diazepam	10	100*

Pentylentetrazole (PTZ) 90 mg/kg s.c.; **12b** and diazepam were administered 30 min before test. Each value represents the mean of 6 mice. *P<0.01 in comparison to vehicle-treated animals (0% of protection)

Table 6. Effect of **12b** in the mouse passive-avoidance test

Treatment	*Dose mg/kg p.o.*	Training session	Retention session
		latency (s)	*latency (s)*
Vehicle + Vehicle		14.1±3.4	98.4±8.8
12b + **Vehicle**	30	16.5±2.9	105.6±10.3
Vehicle +Scopolamine		15.9±3.0	46.3±7.5*
12b + Scopolamine	10	17.5±3.5	53.1±8.0*
12b + Scopolamine	30	17.52±3.1	60.4±9.1*

12b was administered p.o. 30 min before the training session. Scopolamine (1.5 mg/kg) was administered i.p. immediately after the training session. Each value represents the mean of 8 mice. *P<0.01 in comparison to vehicle + vehicle-treated animals (0% of protection)

Table 7. Effect of **12b** in the mouse rota rod test

Treatment	*Dose mg/kg p.o.*	Number of falls in 30 s over time after administration				
		before	*15 min*	*30 min*	*45 min*	*60 min*
Vehicle		5.2±1.6	3.6±0.2	2.0±0.4	0.5±0.1	0±0
12b	30	5.1±1.1	3.3±0.5	2.2±0.3	0±0.2	0±0
Diazepam	10	4.9±0.8	5.2±1.1	6.1±0.8*	5.3±0.6*	4.2±0.9*

Each value represents the mean of 8 mice. *P<0.05 in comparison to vehicle-treated animals.

Table 8. Effect of **12b** in the mouse hole-board test

Treatment	*Dose mg/kg p.o.*	Hole	Board
		number of inspections	*number of movements*
Vehicle		35.6±4.9	75.6±7.4
12b	30	33.5±5.8	81.3±8.1
Diazepam	10	18.8±3.3*	52.4±4.9*

All compounds were administered s.c. 30 min before the test. Each value represents the mean of 8 mice. *P<0.05 in comparison to vehicle-treated animals.

Figure 1.

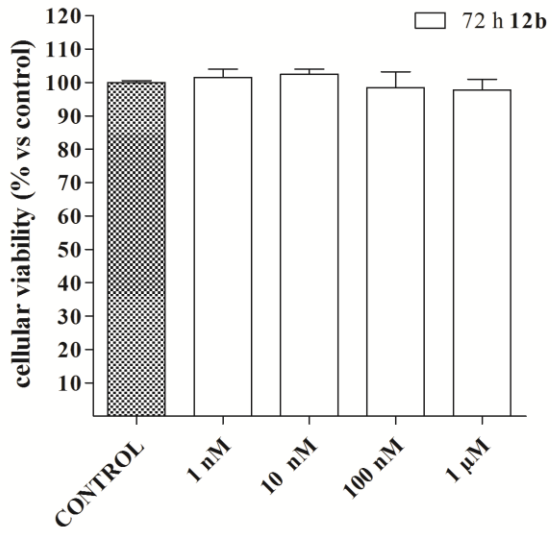


Figure 1. Toxicity of compound 12b. H9-derived NSCs were differentiated for seven days with neurobasal-B27/retinoic acid and then incubated with different concentrations of **12b** (1 nM–1 μM) for 72 h. At the end of the treatments, cell proliferation was measured by MTS assay. The data are expressed as percentages relative to untreated cells (control), which were set at 100%, and are the mean±SEM of three independent experiments, each performed in triplicate. One-way ANOVA followed by a Bonferroni post-test was used to determine statistical significance.

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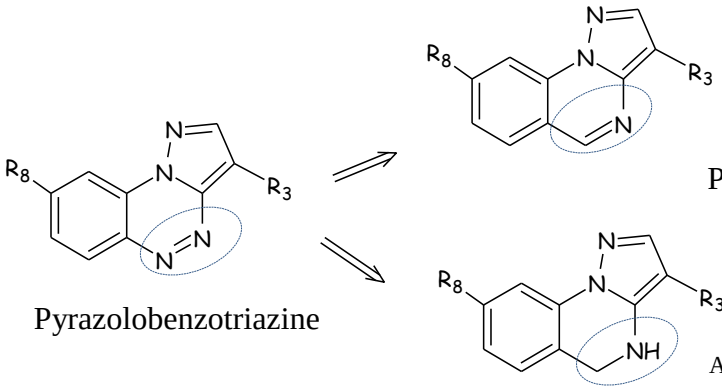

Pyrazoloquinazoline

Pyrazolobenzotriazine

K_i range 0.27-529.3 nM

Anxiolytic-like and antihyperalgesic activity