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PII: S0960-894X(16)30984-2
DOI: http://dx.doi.org/10.1016/j.bmcl.2016.09.042
Reference: BMCL 24266

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 3 August 2016
Revised Date: 14 September 2016
Accepted Date: 15 September 2016

Please cite this article as: Mola, A.D., Gatta, E., Petronzi, C., Cupello, A., Caprariis, P.D., Robello, M., Massa, A., Filosa, R., Synthesis and Pharmacological Evaluation of Functionalized Isoindolinones on GABA-Activated Chloride Currents in Rat Cerebellum Granule Cells in Culture, *Bioorganic & Medicinal Chemistry Letters* (2016), doi: http://dx.doi.org/10.1016/j.bmcl.2016.09.042

Synthesis and Pharmacological Evaluation of Functionalized Isoindolinones on GABA-Activated Chloride Currents in Rat Cerebellum Granule Cells in Culture

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Abstract

A focused N-substituted 3-(2-piperazin-1-yl-2-oxoethyl)-2-(pyridin-2-yl)iso-indolin-1-ones small library was synthesized for modulation of GABA-A receptor function and compared to Zopiclone for the ability to increase GABA-activated chloride currents. All compounds were tested for their effects on GABA-activated chloride currents in rat cerebellar granule cells by use of the whole-cell patch clamp technique. Electrophysiological studies on cultured cerebellar granule cells revealed 3-[2-(4-Methylpiperazin-1-yl)-2-oxoethyl]-2-(5-nitropyridin-2-yl)iso-indolin-1-one (**Id**) as a partial agonist displaying 34% increase of the 10 μM GABA evoked peak chloride currents, antagonized by flumazenil. Moreover, a second group of compounds, with bulky functional groups at N-4 position of piperazine, have shown inverse agonist effects.

The simple synthetic procedure and the possibility of modulating the efficacy of this class of ligands through additional structural modifications pave the way for further development of new molecules as a novel class of compounds able to interfere with benzodiazepine receptors.

GABA-A receptor complexes are associated with, and interact with, membrane channels for chloride ion transport, these complexes are located both within the central nervous system and peripherally.[1,2] There is little doubt regarding the therapeutic possibilities of modulation of GABA-A receptor function.[3,4] These receptors are indeed targets of important drugs such as benzodiazepines, barbiturates, neuroactive steroids and anaesthetics.[5,6] The emerging understanding of the role of different GABA-A receptor subtypes in mediating different physiological functions and pathological processes continues to offer opportunities for novel therapeutics. The benzodiazepine (Bzd) site is an important player in the pharmacology of GABA-A receptors. Clinically used classical 1,4-benzodiazepines such as diazepam have a large spectrum of pharmacological effects such as anxiolysis, sedation/hypnosis, myorelaxation and antiepileptic effects. The 1,5-benzodiazepine clobazam shares the anxiolytic and antiepileptic actions, with less adverse effects. Non benzodiazepine drugs such as zolpidem share some but not all the pharmacological actions. In fact, the hypnotic zolpidem, like zaleplon and indiplon, interacts preferentially with the GABA-A receptors containing, in addition to the β2/3 γ2, the α1 subunit.[3] This receptor subtype mediates the sedative hypnotic action of classical benzodiazepines. Receptors containing α2 or α3 subunit are responsible for the anxiolytic effect.[3] Classical 1,4-benzodiazepines are non selective for the α subunit (a part from the absence of interaction with receptors containing the α6 one) and this explains the presence of a cohort of adverse side effects, when they are used as anxiolytics or antiepileptics.[3]

Zopiclone and its active dextrorotatory stereoisomer eszopiclone, belongs to the class of drugs known as cyclopyrrolones, sedative and anxiolytic drugs widely used in the treatment of insomnia.

They belong to the group of the nonbenzodiazepine drugs which however act as positive allosteric modulators at the benzodiazepine site or a site closely associated with it, within GABA-A receptors.[7-10] Upon binding to the benzodiazepine site, Zopiclone is believed to allosterically

modulate the activity of GABA-A receptors by increasing transmembrane conductance of chloride ions,[10,11] thus stabilizing neuronal membrane potentials and dampening excitatory input.

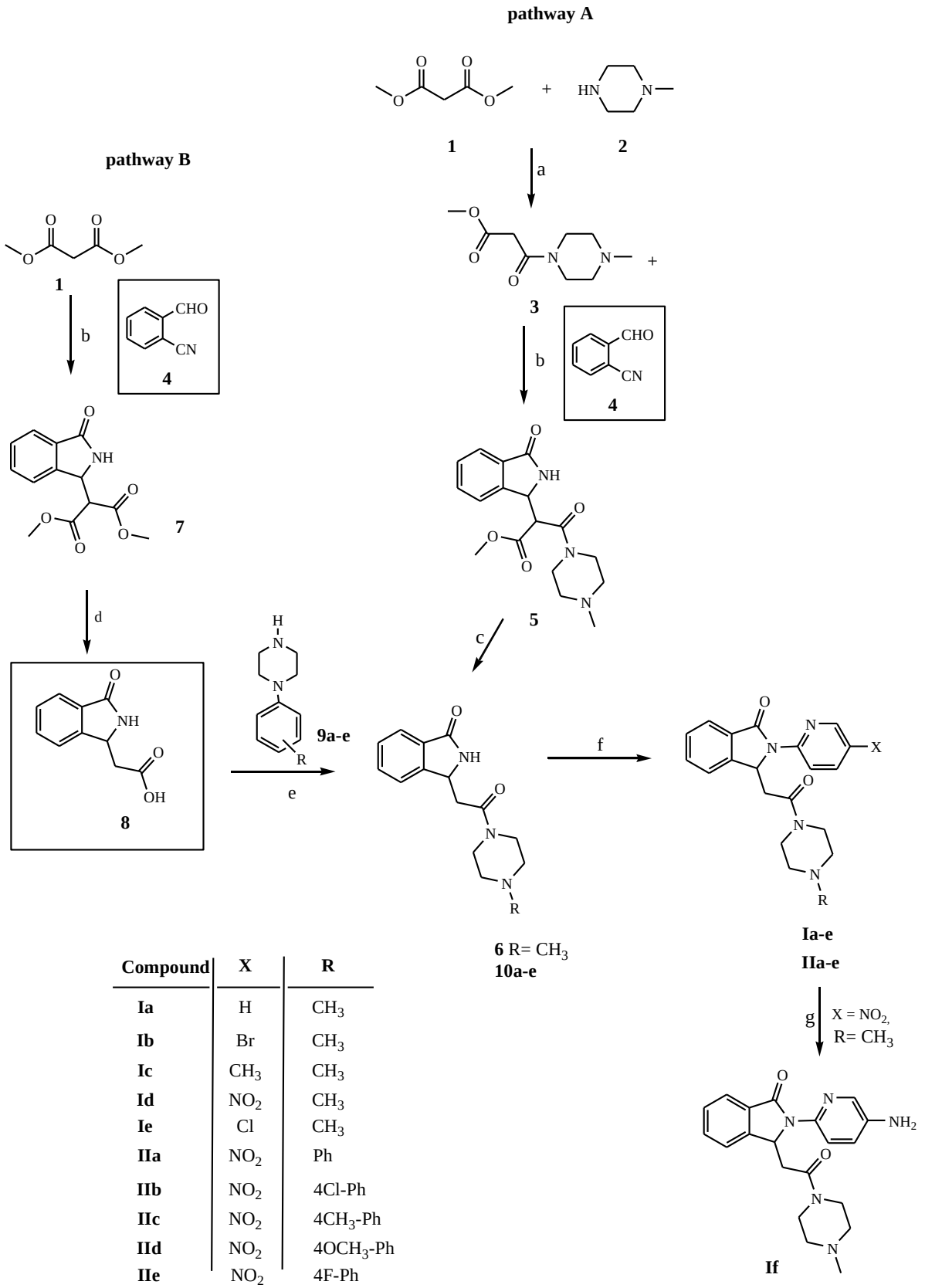
Much of the interest about Zopiclone is due to the fact that, at difference from classical 1,4-benzodiazepine, it has an anxiolytic and hypnotic effect without negative side effects such as addiction[12,13]. Also in this case, GABA-A receptor subtype selectivity is thought to underlie such characteristics. In general, studies in this respect only partially confirm this concept.[10,11]

However, the study by Petroski et al.[14] indicates a greater potency of Zopiclone at α1β2γ2 and α5β2γ2 in the respect of α2β2γ2 and α3β3γ2 receptor subtypes. Moreover, in contrast to Zopiclone, pagoclone[15] and pazinaclone produce anxiolytic effects with little or no sedative or amnestic actions at low doses. This is because these compounds are subtype-selective drugs which bind primarily to the α2/α3 subtypes of the GABA-A receptor which are responsible for the anti-anxiety effects of drugs of this kind, but have relatively little efficacy at the α1 subtype which produces the sedative and memory loss effects.[3]

In the present article, we describe the synthesis and the pharmacological evaluation of a focused library with isoindolinone backbone and the evaluation of their effects on GABA-activated chloride currents by use of the whole-cell patch clamp technique activity in rat cerebellar granule cells, a useful model for studying new benzodiazepine site ligands.[16]

In these cells in culture we previously evaluated the following distribution of GABA-A receptors expressed on the plasma membrane and contributing to the rapidly desensitizing (peak) chloride current: 25 % α6 β2/3 incomplete receptors, 45 % α1 β2/3 γ2, and 30 % α6 β2/3 γ2 + α1 α6 β2/3 γ2 subtypes.[17] Tandem reactions of 2-formyl benzonitriles **4** (2-cyano benzaldehydes) are among the most powerful strategies for the synthesis of 3-substituted isoindolinones.[18] Taking advantage from these very convenient and operational simple one-pot methodologies, we envisioned effective routes to the designed derivatives **Ia-e** and **IIa-e** object of the present investigation (**Scheme 1**). In an early approach, the *N*-methyl piperazine scaffold was introduced reacting the mono-malonoamide derivative **3** with **4**, followed by Krapcho decarboxylation (pathway A).[19] Then, Cu(I)

NH-arylation of the resulting lactam[20] **6** with 5-substituted 2-iodopyridines led to the desired products **Ia-e**. Hydrogenation of nitro group in **Ie** gave in almost quantitative yield compound **If**, with amino group on the pyridine ring. However, the described approach allows a linear synthesis of the target compounds, not particularly convenient to build up a diversified library to effectively tackle the major challenges in medicinal chemistry. For these reasons, recently, we focused on the obtaining of a common key intermediate to be employed in a divergent approach to diverse functionalized isoindolinones (pathway B). In this respect, tandem reaction of dimethyl malonate with 2-cyanobenzaldehyde[21] and the following HCl 6M decarboxylation furnished the isoindolinone acetic acid **8** in very high yields, a synthetic sequence also applicable for large scale synthesis. This compound was, then, subjected to the amide coupling with different 4-substituted arylpiperazines **9a-e**, in the presence of EDC·HCl (*N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimidehydrochloride) and HOBT (1-hydroxybenzotriazole hydrate).[22] The obtained products **10a-e** were arylated in an improved version of the Cu(I) NH arylation, giving the target compounds in very good overall yields.



Compound	X	R
Ia	H	CH_3
Ib	Br	CH_3
Ic	CH_3	CH_3
Id	NO_2	CH_3
Ie	Cl	CH_3
IIa	NO_2	Ph
IIb	NO_2	4Cl-Ph
IIc	NO_2	$4CH_3$-Ph
IId	NO_2	$4OCH_3$-Ph
IIe	NO_2	4F-Ph

Scheme 1

Reagents and conditions. a) Toluene, reflux 48h; b) K_2CO_3, CH_3CN, r.t; c) LiCl, DMF/H_2O, MW; d) HCl 6M, 100 °C 1h; e) EDC·HCl, 5-HOBt, 4-substituted phenylpiperazines, Et_3N, THF, r.t, 16h.; f) 5-substituted 2-iodopiridines, CuI, K_3PO_4, *N*,*N*^{1} dimethyl-ethylen-diamine, dry toluene, 100°C (for Ia-e) or dry dioxane 80 °C (for IIa-e) , 24h; g) H_2, Pd/C 5%, MeOH, 24h.

We synthesized isoindolin-1-one derivatives in racemic form, different substituents, chloro, bromine, methyl, and nitro groups to the p-position in pyridine core were introduced, and in order to increase the water solubility of the compounds as hydrochloride salts, we introduced a substituent at the 3-position in the form of a 2-(4-methyl-1-piperazinyl)-2-oxoethyl group also present in the lead compound. A part from compounds If and IIc which show limited solubility in DMSO, all the compounds were fully soluble in this solvent, slightly soluble in ethanol and insoluble in water. Solubility in phosphate buffer (pH 3.2) was very high due to salt formation by protonation of piperazine ring.

First of all, the effect of the lead compound Zopiclone on the peak chloride current elicited by 10 μM GABA (a GABA concentration close to the EC50 for the peak current activation in this model) [23] was determined at the concentration of 1 μM. This concentration is already in the saturation range of zopiclone positive allosteric effect on recombinant α1 β3 γ2 receptor subtype[11] and in our model the α1 β2/3 γ2 subtype is the only one bearing a benzodiazepine site.[16]An augmentation of the peak current by 58 ± 7 % resulted (Table I). In these experiments all the zopiclone analogue tested were at 1 μM concentration, as an index of their efficacy (only If and IIc were at respectively 0.47 and 0.58 μM, as the maximal concentrations possible due to limited solubility in the media used). As shown in **Table I** the structural simplification of the lead demonstrated a decrease in activity (**Ia** vs Zopiclone: $p^z < 0.01$) and the introduction of electron-releasing and electron-withdrawing groups in *p*-position (**Ib** and **Ie)** demonstrated no potentiation of the current in the respect of **Ia**.

Besides, compound **Ic** exerted an increment in efficacy versus **Ie** ($p < 0.05$) and furthermore, the 4-nitro analog **Id** demonstrated (**Fig. 2**) maximum potentiation 34% above control.

Interestingly, reduction of NO_2 group led to an inverse agonist **If** (**Table I**).

Modification of the terminal methyl group on piperazine substituent of **Id** into a bulky substituent was next examined. (**Table II**).

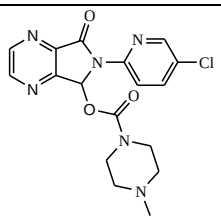
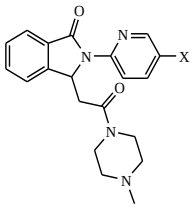
Compounds	X	*E%*	Statistical significance (Student's t-test)
 **Zopiclone**		58 % ± 7	p^a <0.01 p^b<0.01
			
Ia	H	20% ± 3	p^a <0.01 p^b<0.01 p^z <0.01
Ib	CH_3	21% ± 3	p^a <0.01 p^b<0.05
Ic	Br	26 % ± 3	p^a <0.01 n.s.^{b}
Id	NO_2	34% ± 4	p^a <0.01
Ie	Cl	18% ± 2	p^a <0.01 p^b<0.01
If	NH_2	-23% ± 2	p^a <0.01

Table I

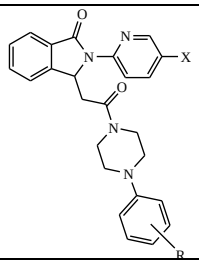
Compounds	*R*	*X*	*E%*	**Statistical significance (Student's t-test)**
				
IIa	H	NO_2	-27% ± 2	p^a <0.01
IIb	4-chloro	NO_2	-36% ± 4	p^a <0.01
IIc	4-methyl	NO_2	n.s[a]	
IId	4-methoxy	NO_2	-21% ± 6	p^a<0.01
IIe	4-fluoro	NO_2	n.s[a]	

Table II

Here, it is interesting to underline that in compound **IIa** the replacement of methyl with phenyl in the piperazine group caused a transition from agonistic to inverse agonistic activity. This change is displayed by compounds **IIb** (**Fig.3**) and **IId** as well and indicates that most probably the π–π interaction with receptor protein is highly required to display an inverse agonist profile. As shown in **Fig.'s 1-3**, both the agonistic (first series of compounds) and the inverse agonist activities (second series of compounds) were reversed by the Bzd site antagonist flumazenil. These reversions were repeated on three different experiments. Zopiclone is a non-benzodiazepine hypnotic agent able to bind at or near benzodiazepine sites on GABA-A macromolecular receptor complexes[8,9]. It is part of a relatively new generation of drugs able to produce comparable anxiolytic effects with less sedation, muscle relaxation, or addictive potential[24,25].

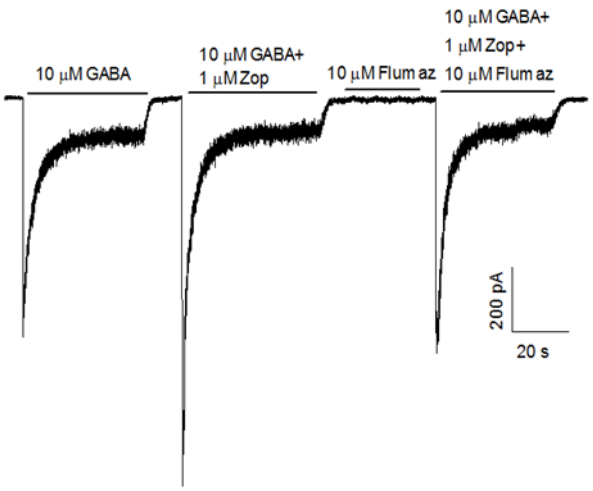


Fig.1

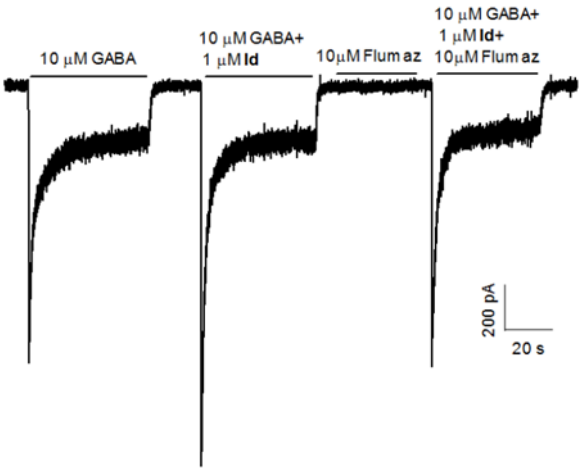

10 μM GABA
10 μM GABA+
1 μM Id
10μM Flumaz
10 μM GABA+
1 μM Id+
10μM Flumaz
200 pA
20 s

Fig.2

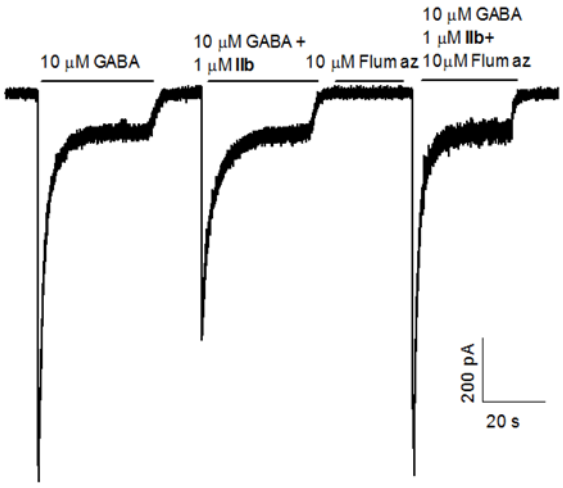

10 μM GABA
10 μM GABA +
1 μM IIb
10 μM Flumaz
10 μM GABA
1 μM IIb+
10μM Flumaz
200 pA
20 s

Fig.3

Racemic Zopiclone and its more active stereoisomer S-Zopiclone augment chloride currents due to α1β2γ2, α2β2γ2 and α3β3γ2 GABA-A receptor subtypes[10]. According to Petrovski et al. (2006)[14], its maximal potency is versus α1β2γ2 and α5β2γ2 receptor subtypes. This might explain its specificity as an hypnotic, since α1βxγ2 receptor subtypes are those mediating sedation by Bzd agonists, whereas anxiolysis involves receptors containing α2 or α3 subunits[4,26].

Along this line, it seems interesting to find new drugs which may have an even greater specificity in terms of interaction with GABA-A receptor subtypes and with improvement in metabolic stability. The metabolism of Zopiclone, in fact is rapid, complex, and differs among species; of the more than ten metabolites of the compound that have been identified, however, only two reportedly exhibit pharmacological activity in humans: N-desmethyl zopiclone and zopiclone-N-oxide. It is interesting to note that approximately 50% of the administered dose is decarboxylated to inactive metabolites. Moreover, Zopiclone has also an affinity for acetylcholine muscarinic receptors[27], consequently, administration results in adverse effects such as, but are not limited to, drymouth, thirst, slowing and acceleration of the heart, dilated pupils, blurred vision, restlessness, fatigue, headache, hallucinations and delirium.

In our research interested in synaptic transmission in the central nervous system, and particularly amino acid neurotransmission [28-30], we synthesized and tested new compounds with isoindolinone skeleton and with 2-oxoethyl group instead of ester group. It is interesting to note that the major metabolic routes, affecting more than 50% of dose of the parent compound Zopiclone, involve the transformation to the inactive decarboxylated metabolite, taking in mind that, in general, carbamate is more susceptible to hydrolysis than amides[31], we replaced it to the relatively metabolically stable amidic group.

In the present study and model, the two series of zopiclone related new compounds were tested for their effects on the GABA activated rapidly desensitizing (peak) current component. In previous studies it was determined that this component is due to GABA-A receptors of the α6 β2/3 incomplete receptors, α1 β2/3 γ2, and α6 β2/3 γ2 + α1 α6 β2/3 γ2 subtypes[17]. Since the effects (

both positive and negative allosteric modulation) by the new compounds tested are reversed by the benzodiazepine antagonist flumazenil, only the α1β2/3γ2 GABA-A receptor subtype is involved. In fact, the α6 containing receptors are not affected by benzodiazepines.[32,33]

Within the first group of compounds **Ia-e**, the most active was found to be **Id**, with a very interesting 34% increment of chloride peak current activated by 10 μM GABA.

Thus, since in our model the α1β2/3γ2 receptor subtype represents 45 % of the total of the GABA-A receptors generating the peak current, the actual effect on the affected receptors can be estimated as 2.20 times the one evaluated on the basis of the difference in the peaks with versus without the drugs studied. This is exactly what we experimentally found for diazepam and clobazam erasing by furosemide the contribution of α6 containing receptors to the peak current.[34]

This enhancement of chloride current was antagonized by flumazenil, thus confirms the involvement of the benzodiazepine site. The lower activity vs Zopiclone is probably due to the absence in **Id** of one of the two nitrogens present in the Zopiclone pyrazine group that acts as an electron donor for the H1 hydrogen bond within the receptor[7,9]. The absence of the N atom in compound **Id** may decrease the binding to the receptor and the pharmacological effect.

It is remarkable that passing from **Id** to **If** the only change is a $-NH_2$ group instead of $-NO_2$, however the pharmacological activity changes completely from agonist (+ 34 %) to inverse agonist (-23 %).

Referring to the second group of compounds **IIa-e**, they share a nitro group on the pyridine ring with bulky groups at the N in position 4 of the piperazine ring and behave as inverse agonists. An explanation of the drastic change of activity may be found, as described by Borea et al. (1987)[7], in the interaction between agonists/antagonists/inverse agonists and the Bzd site.

According to this, there are two lipophilic interaction points whose engagement determines the positive allosteric (agonistic) activity: **AG1** and **AG2.** Within this scheme, Zopiclone interacts with **AG1** with the 3-Cl-piridine ring and with **AG2** with the piperazine ring, this results in an agonistic activity. The introduction of bulky groups on the nitrogen in position 4 in the piperazine ring may

push piperazine and its substituent out of the **AG2** interaction point and laterally towards a different interaction point, which Borea et al.[7] defined as **IAG**, critical for the inverse agonist activity like that of β-carboline structure derivatives.

This possibility can be further tested by studying the effects of different substituents on the piperazine ring, As an example, introducing a cyclohexane would permit to investigate whether a bulky but more flexible and non aromatic structure maintains or not the transition to negative allosteric modulation in the resulting molecule.

In conclusion, a novel series of agonists at the Bzd binding site of the GABA-A receptor based on modifications of a known template were described. The optimization work surprisingly yielded both agonists and inverse agonists depending on the substitution patterns.

Currently, further work is ongoing to evaluate compound **Id** in several different in vivo models with the aim of obtaining a safer pharmacokinetic profile.

Acknowledgements

This work was supported by the grant PON 03 PE_00060_07 (MIUR) Sviluppo pre-clinico di nuove terapie e di strategie innovative per la produzione di molecole ad azione farmacologica.

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Legends to Schemes, Tables and Figures

Scheme 1: Pathway of synthesis of compounds **Ia-f** and **IIa-e**

Table I: Structures of the first series of compounds, **Ia-f** and the lead compound Zopiclone and percent stimulations of the chloride current elicited by 10 μM GABA. The data are reported as mean E % (see main text) ± SEM. Student's t-test. p^a: compound plus GABA vs GABA alone; p^b: compound vs **Id**; n.s.^{b}: not significant, compound vs **Id**; p^z: compound vs **Zopiclone**. The compounds were tested at 1 μM, except **If**, which for solubility limit reason was tested at 0.47 μM. The numbers of samples were: **Zopiclone** (N=5); **Ia** (N=7); **Ib** (N=6); **Ic** (N=6) **Id** (N=8); **Ie** (N=8); **If** (N=15).

Table II: Structures of the second series of compounds, **IIa-e** and percent changes of the chloride current elicited by 10 μM GABA. The data are reported as mean E % (see main text) ± SEM. Student's t-test. p^a: Compound plus GABA vs GABA alone; n.s.^{a}: not significant, compound plus GABA vs GABA alone. The compounds were tested at 1 μM, except **IIc** which for solubility limit reason was tested at 0.58 μM. The numbers of samples were: **IIa** (N=19); **IIb** (N=14); **IIc** (N=19); **IId** (N=6); **IIe** (N=24).

Figure 1: Chloride currents evoked by (from left to right): 10 μM GABA alone, Zopiclone 1 μM plus 10 μM GABA, 10 μM plain flumazenil and Zopiclone 1 μM plus 10 μM GABA in the presence of 10 μM flumazenil.

Figure 2: Chloride currents evoked by (from left to right): 10 μM GABA alone, **Id** 1 μM plus 10 μM GABA, 10 μM plain flumazenil and **Id** 1 μM plus 10 μM GABA in the presence of 10 μM flumazenil.

Figure 3: Chloride currents evoked by (from left to right): 10 μM GABA alone, **IIb** 1 μM plus 10 μM GABA, 10 μM plain flumazenil and **IIb** 1 μM plus 10 μM GABA in the presence of 10 μM flumazenil.

Graphical Abstract

Synthesis and Pharmacological Evaluation of Functionalized Isoindolinones on GABA-Activated Chloride Currents in Rat Cerebellum Granule Cells in Culture

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