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The discovery and unique pharmacological profile of RO4938581 and RO4882224 as potent and selective $GABA_A$ α5 inverse agonists for the treatment of cognitive dysfunction

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ABSTRACT

Lead optimisation of the imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzodiazepine class led to the identification of two clinical leads [RO4882224 (**11**) and RO4938581 (**44**)] functioning as novel potent and selective $GABA_A$ α5 inverse agonists. The unique pharmacological profiles and optimal pharmacokinetic profiles resulted in in vivo activity in selected cognition models.

GABA is the major inhibitory neurotransmitter in the CNS and of three existing receptor families ($GABA_A$, $GABA_B$ and $GABA_C$) the $GABA_A$ receptor has attracted most interest as a therapeutic target for treating central disorders. Many anxiolytic, sedative and hypnotic drugs that bind to the benzodiazepine site of the $GABA_A$ receptor increase the affinity for GABA and thereby enhance the chloride conductance. Ligands binding to the same site and producing the opposite effect, that is, reduction of GABA affinity and chloride conductance have been termed 'inverse agonists'.[1]

In contrast, extensive pharmacological evidence exists in animals and humans that $GABA_A$ inverse agonists enhance cognitive functions.[2,3]

There is a large unmet medical need for treatment of cognitive deficits in ageing western societies with millions of patients suffering from Alzheimer's disease and other types of dementias. Currently available therapies are based either on cholinesterase inhibition (e.g., donepezil)[4] or on NMDA receptor antagonism (memantine).[5] However, the effectiveness of current therapies is modest and cholinesterase inhibitors suffer from mechanism-related side effects.[3,4] There is a clear need for a novel therapy with improved efficacy and better tolerability. Therefore, inhibition of $GABA_A$ receptor function remains an attractive alternative, provided compounds are selective for the receptor subtype mainly involved in memory formation (Table 1).[3]

Screening of our in house corporate collection led to a 'benzodiazepine-rich' hit list from which we selected an imidazo[1,5-*a*]-pyrimido[5,4-*d*]benzazepine class to follow up on.[6] Preliminary SAR generally displayed typical features of benzodiazepine structures in terms of binding affinity at the $GABA_A$ α5 receptor subtype but missing the desired inverse agonism. However, SER (structure–efficacy relationship) at the $GABA_A$ α5 receptor subtype led to the identification of the imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzo-diazepines **II** (Scheme 1) as potent and efficacious inverse agonists with binding selectivity potential.[7]

Table 1
Characteristics of efficacy at the $GABA_A$ subtypes[3]

Agonism	$GABA_A$ αxβ3γ2	Inverse agonism
Cognitive deficits	α5	Cognitive enhancement
Sedative	α1	Proconvulsant
Anxiolytic	α2	Anxiogenic
Anxiolytic	α3	Anxiogenic

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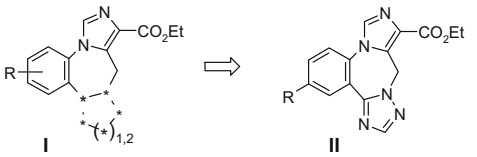
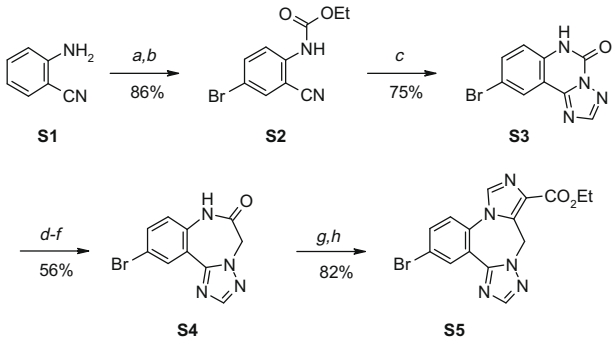


Scheme 1. From HTS to a promising lead series.



Scheme 2. Reagents and conditions: (a) KBr, $NaBO_3$, AcOH, 15 °C; (b) ethyl chloroformate, reflux; (c) formylhydrazine, NMP, 160 °C; (d) NaOH, ethylenglycol, 100 °C; (e) chloroacetylchloride, AcOH, 15 °C; (f) NaOH, dioxane, 25 °C; (g) 1,2,4-triazole, $POCl_3$, Hünigs base, MeCN, 90 °C; (h) ethyl isocyanoacetate, KOtBu, DMF, −50–0 °C.

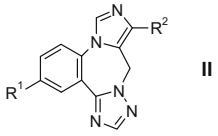
The compounds from the described imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzodiazepine class carrying an ester functionality in general display a very high affinity (nM levels) to the $GABA_A$ α5 subtype receptor, however, the subtype binding selectivity (vs α1, α2 and α3) and inverse agonism efficacy was rather low (e.g., **3**, Table 2). In addition, the ester functionality with its inherent susceptibility to esterases was not suitable for daily dosing due to a short half-life.[6]

During our attempts to discover a suitable ester replacement we explored a large diverse set of surrogates with a wide range of substituents at the 4-position (R^2) of the imidazo core spanning from no substitution (R^2 = H), to small alkyl-substituents through to heteroaromatic moieties (Table 1 depicts a selection). Most of these surrogates had low nM affinity towards the target with the exception of the ethylsulfonyl derivative **6**. Unfortunately, the efficacy range was only between medium/weak inverse agonism to antagonism, and in addition a low $GABA_A$ subtype selectivity was observed. The unsubstituted, imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzodiazepine **1** combines the desired target affinity and full inverse agonism (K_i = 6.4 nM, −37%) but unfortunately displayed an inverted binding selectivity against the $GABA_A$ α2 and $GABA_A$ α3 subtype receptors.

Compounds **10** and **11** bearing, as R^2, a methyl-group or a chlorine atom, respectively, have been selected for further investigation due to their high affinity (**10**: K_i = 3 nM, **11**: K_i = 2 nM) to the target receptor, a moderate to excellent subtype binding selectivity (**10**: 88-fold against $GABA_A$ α3) as well as their full inverse agonism efficacy (**10**: −41%, **11**: −40%).

We have developed a flexible synthesis of tetracyclic imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzodiazepines is based on the sequential construction of the individual ring-systems (Scheme 2). This begins with a selective bromination[8] of the commercially available 2-aminobenzonitrile **S1** followed by carbamate formation, affording intermediate **S2** ready for the first cyclisation reaction by treatment with formylhydrazine at high temperature. The putative carbamoyl-hydrazone intermediate drives the reaction in an intramolecular manner obtaining the tri-

Table 2
Binding and efficacy profile of analogues of **II**



Compd	R^1	R^2	Affinity $GABA_A$ α5β3γ2a K_i (nM)	Selectivity: $\frac{K_i\ (nM)\ \alpha x\beta 3\gamma 2^a}{K_i\ (nM)\ \alpha 5\beta 3\gamma 2^a}$			Efficacy $GABA_A$ α5β3γ2 receptors[b] (%)
				Versus α1	Versus α2	Versus α3	
1	H	H	6.4[c]	15[c]	0.05[c]	0.06[c]	−37
2	F	H	10[c]	12[c]	4[c]	3[c]	−45
3	F	CO_2Et	0.2[c]	1[c]	1[c]	1[c]	−16
4	F	$C(O)NHCH_2CCH$	68	4	1	1	−35
5	Cl	$C(O)CH_2CH_3$	2.6[c]	12[c]	11[c]	8[c]	−38
6	Cl	$SO_2CH_2CH_3$	215	4	>15	4	−23
7	F	$CF_2CH_2CH_3$	3.2	5	3	2	−27
8	Cl	nPr	1.6	3	5	3	−35
9	Cl	cPr	9.4	6	7	5	−21
10	Cl	Me	3.0	6	72	88	−41
11	Cl	Cl	2.0	7	12	7	−40
12	Cl	CN	6.4[c]	7[c]	11[c]	9[c]	−31
13	Cl	2-Methyl-pyridin-6-yl	31	11	11	8	−12
14	F	5-Methyl-[1,2,4]-oxadiazol-3-yl	0.2	3	5	3	−12
15	Br	Thiophen-2-yl	4.0	27	22	16	−10[d]
16	Br	1-Methyl-1H-pyrazol-5-yl	9.7	26	35	22	−33[d]

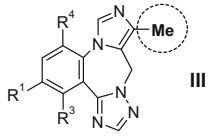
[a] Cloned human receptor sub-units were expressed in HEK293 cells (transient transfected) for ^{3}H-flumazenil α1, α2, α3 and α5 binding and the K_i values are calculated as mean values of duplicate determinations.

[b] Cloned rat receptor sub-units were expressed in HEK293 cells for electrophysiology measurements. Efficacy is determined as the percentage change of a submaximal (EC_{10}) response to GABA.

[c] Cloned human receptor sub-units were expressed in insect SF9 cells for ^{3}H-flumazenil α1, α2, α3 and α5 binding and the K_i values are calculated as mean values of duplicate determinations.

[d] Cloned human receptor sub-units were expressed in *Xenopus laevis* oocyts for electrophysiology measurements. Efficacy is determined as the percentage change of a submaximal (EC_{10}) response to GABA.

Table 3
Binding and efficacy profile of analogues of **III**

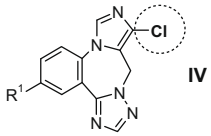


III

Compd	R^1	R^3	R^4	Affinity $GABA_A$ α5β3γ2a K_i (nM)	Selectivity: $\frac{K_i\ (nM)\ \alpha x\beta3\gamma2^a}{K_i\ (nM)\ \alpha5\beta3\gamma2^a}$			Efficacy $GABA_A$ α5β3γ2 receptors[b] (%)
					Versus α1	Versus α2	Versus α3	
10	Cl	H	H	3.0	6	72	88	−41
17	H	H	H	2.6	16	6	4	−44
18	F	H	H	3.0	6	3	4	−38
19	Br	H	H	6.2	22	30	21	−42
20	I	H	H	4.4	42	60	29	−57[d]
21	Me	H	H	9.2	11	34	23	−37[d]
22	CHF_2	H	H	2.2	29	62	30	−45[d]
23	CF_3	H	H	18	29	78	29	−32[d]
24	OMe	H	H	7.7	11	31	18	−45[d]
25	$OCHF_2$	H	H	1.8	58	53	33	−43[d]
26	OCF_3	H	H	50	10	29	24	−41
27	H	H	F	64	12	12	5	−50[d]
28	H	F	H	1.0	5	13	6	−42[d]
29	H	Cl	H	1.2	0.6	2	1	−45[d]

[a,b,d]See Table 2.

Table 4
Binding and efficacy profile of analogues of **IV**



IV

Compd	R^1	Affinity $GABA_A$ α5β3γ2a K_i (nM)	Selectivity: $\frac{K_i\ (nM)\ \alpha x\beta3\gamma2^a}{K_i\ (nM)\ \alpha5\beta3\gamma2^a}$			Efficacy $GABA_A$ α5β3γ2 receptors[b] (%)
			Versus α1	Versus α2	Versus α3	
11	Cl	2.0	7	12	7	−40
30	H	0.3	13	5	2	−41
31	F	0.3[c]	3[c]	4[c]	3[c]	−33
32	Br	1.4	24	18	7	−48
33	CN	2.4	15	16	5	−27
34	Me	1.8	11	23	12	−37
35	CHF_2	1.1	19	36	15	−42
36	cPr	1.2	23	15	16	−47
37	Ph	6.3	4	5	8	+8
38	NHcPr	2.2	10	43	36	−37
39	NHAc	2.9	14	19	10	−34
40	NHC(O)cPr	16	2	6	5	−48[d]
41	$NHSO_2Me$	54	7	5	5	−36[d]
42	1*H*-Imidazol-1-yl	13	1	2	1	−7

[a–d]See Table 2.

Table 5
Binding and efficacy profile of analogues of **II–IV** (structure and R^1, R^2 see Tables 2–4)

Compd	R^1	R^2	Affinity K_i (nM) GABA A αxβ3γ2 receptors[a]				Efficacy GABA A αxβ3γ2 receptors[b] (%)			
			α5	α1	α2	α3	α5	α1	α2	α3
11	Cl	Cl	2.0	15	24	14	−40	−3	−9	−11
10	Cl	Me	3.0	18	219	265	−41	−11	−2	+4
43	Cl	CHF_2	5.6	53	81	48	−18	−20	+2	+21
32	Br	Cl	1.4	33	24	8.8	−48	−13	−25	−14
19	Br	Me	6.1	136	186	132	−42	−9	nt	nt
44	Br	CHF_2	4.6	174	185	80	−35	−3	−4	+2

[a,b]See Table 2.

azole **S3** in a yield of 75%. Hydrolysis of **S3** followed by treatment with chloroacetylchloride completes the diazepine-ring system **S4**. Imidazole formation using standard procedures,[9] was performed after activation of **S4** as an iminoyl-triazolide,[10] giving the desired product **S5** (imidazo-triazolobenzodiazepine) in high yield.

Table 6
Properties and pharmacokinetic parameters (in rat)

Compd	Solubility[a] (mg/L)	Log *d*[b]	Pe[c]	Cl[d] (rat/ human)	Half life $t_{1/2}$ (h)	Cl[e]	Vss (l/kg)	*F* (%)	B/P[f]
11	13	2.0	8.9	0/0	3.4	7.2	1.9	52	0.6
10	27	1.8	6.5	11/16	0.6	12.4	0.5	81	0.2
43	78	1.6	7.6	0/0	7.7	0.6	0.4	80	nt
32	9	2.2	7.0	1/2	1.6	9.7	1.3	56	0.0
19	58	2.0	6.3	3/13	0.9	3.8	0.3	87	nt
44	19	1.9	7.0	0/0	4.9	2.9	1.2	89	0.4

[a] Lyophilisation solubility assay (LYSA) (mg/L), for measurement details see Ref. 12.
[b] At pH 7.4.
[c] Permeation coefficient (parallel artificial membrane permeability assay, PAMPA) (10^{-6} cm s^{-1}), for details see Ref. 13.
[d] Microsomal clearance (mL/min/mg protein).
[e] Clearance (mL/min/kg).
[f] Brain/plasma ratio.

The ester moiety of **S5** was then transformed into a diverse set of derivatives[11] while the bromo substituent opens up complementary possibilities for further elaboration. Various substituted starting materials (e.g., 5-fluoro-2-aminobenzonitrile, 5-methoxy-2-aminobenzonitrile) could be transformed into the corresponding products following the same procedures (Scheme 2, steps b–h).

As depicted in Tables 3 and 4, a detailed study of variations at the R^1 position revealed that rather small substituents such as halogen, small alkyl or alkoxy substituents (amino substituents were of less interest) are preferred (see Tables 3 and 4) in order to combine high target affinity with subtype binding selectivity (e.g., **20**: $R^1 = OCHF_2$, Table 2, or **36**: R^1 = cPr, Table 3) although subtle changes (**21**: $R^1 = OCF_3$, Table 2) led to a sharp drop in affinity. The substitution position R^1 versus R^3 and R^4 clearly plays a crucial role demonstrated by compound **27** (R^4 = F, vs **17**, Table 2) which dropped 25-fold in $GABA_A$ α5 affinity and compound **24** (R^3 = Cl, vs **17**, Table 2) completely loses subtype binding selectivity. Notably, both compounds **17** and **24** demonstrated full inverse agonism with −50% (**17**) and −45% (**24**) efficacy at the $GABA_A$ α5 receptor subtype.

The imidazo methyl- or chloro-substituted series **III** and **IV** always displays high efficacy pointing towards a separation of efficacy (controlled by R^2 in addition to the inherent efficacy of the imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzodiazepine core) and subtype binding selectivity (controlled by R^1, R^3 and R^4) with only few exceptions when R^1 is of aromatic (**37**, Table 4) or heteroaromatic (**42**, Table 4) nature.

Taking into account the balanced profile of target binding affinity and degree of inverse agonism as well as subtype binding and functional selectivity achieved the most interesting compounds turned out to be the corresponding (R^1 = Cl, Br) chloro- (**11**, **32**) and methyl-substituted (**10**, **19**) analogues and the full profiles of binding and efficacy at all relevant $GABA_A$ α subtypes are depicted in Table 5. Complimentary to the receptor parameters discussed above, also molecular properties were regularly determined and were found to be in a typical range for CNS compounds (Table 6), except for microsomal clearance values for the R^2 = Me compounds **10** and **19** that are at the higher end of the desired range. In order to increase the metabolic stability fluorinated derivatives were prepared and to our delight the corresponding $-CHF_2$ analogues (**43**, **44**) were found to be similar in terms of potency and efficacy, and maintained the corresponding binding and functional selectivity and also displayed a much higher degree of metabolic stability in the microsomal clearance assay (Table 6) which was mirrored in a lower clearance in in vivo rat PK studies.

Based on the overall pharmacokinetic profile (half life, bioavailability and brain/plasma separation) compounds **11** and **44** have been selected for an in depth profiling in behavioural pharmacology and were found to be in vivo active in a wide range of tests. Notably, compounds **11** and **44** were found to enhance hippocampal long-term potentiation (LTP) in vitro (mouse) using a stimulus paradigm found to be sensitive to allosteric modulators of GABA receptors.[14] Moreover, both compounds reversed a scopolamine-induced working memory impairment in the delayed match to position (DMTP) task (**44**: 0.3–1 mg/kg po, **11**: 1 mg/kg, Fig. 1) and a diazepam-induced spatial learning impairment (**44**: 1–10 mg/kg po) in rats. In addition, **44** improved executive function in an object retrieval task in cynomolgus monkeys (3–10 mg/kg po).[15] Most importantly, **44** showed no anxiogenic potential in the elevated plus maze and social approach avoidance tests in rats and no pro-convulsive potential in an audiogenic seizure model in mice, underlying the critical importance of subtype binding and functional selectivity achieved.

DMTP is a test of spatial working memory, which is dependent on hippocampal function.[16] Two groups of 11 male Lister hooded rats were pre-trained to asymptotic performance in the DMTP task (1–24 s delay intervals) and were tested using a Latin squares design (statistics: two factor repeated measures analysis of variance (ANOVA) followed in significant cases (p <0.05) by post-hoc Newman Keuls test). Scopolamine (0.03 mg/kg sc) significantly reduced percent correct responses in a delay-dependent manner (p <0.01 at 16 and 24 s; Fig. 1), indicating a selective impairment of working memory. RO4938581 significantly (p <0.01) reversed the scopolamine impairment at 0.3 and 1 mg/kg at both delay intervals to control (vehicle) levels, whereas at 0.1 mg/kg there was a partial but significant (p <0.05) reversal at the 24 s delay only (Fig. 1a). RO4882224 at 1 mg/kg po had no effect alone, but significantly

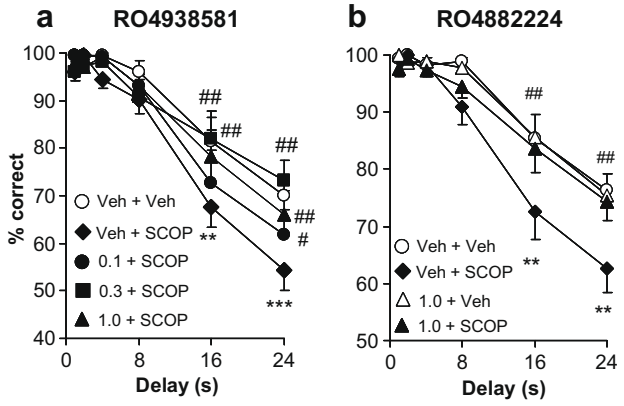
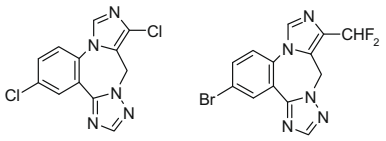


Figure 1. The effect of (a) RO4938581 at 0.1, 0.3, 1 mg/kg po and (b) RO4882224 at 1 mg/kg po alone and versus a scopolamine-induced working memory impairment in the DMTP task Data are presented as mean percent correct responses ± SEM at each delay interval(s). Statistics: $^{**}p$ <0.01, $^{***}p$ <0.001 scopolamine-treated versus vehicle group; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 RO + scopolamine-treated versus scopolamine group.



11 44

Scheme 3. Clinical candidates RO4882224 (**11**) and RO4938581 (**44**).

(p <0.01) reversed the scopolamine-induced impairment at 16 and 24 s delay intervals (Fig. 1b).[17]

Supported by these results the imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzodiazepines **11** and **44** have been selected as clinical candidates for further development (Scheme 3).

An ambitious lead optimisation programme delivered, by extensive structure–activity- and structure–efficacy-relationship work, within the imidazo[1,5-*a*][1,2,4]-triazolo[1,5-*d*][1,4]benzodiazepine chemical class, potent inverse agonists at the $GABA_A$ α5 receptor sub type, which feature both binding and functional selectivity in a unique pharmacological profile. This dual binding and functional selectivity offers an ideal profile for cognition-enhancing effects without the unwanted side effects associated with activity at other $GABA_A$ receptor subtypes. Furthermore, the in vivo DMTP data further support the potential of $GABA_A$ α5 receptors as a valuable target for cognition-enhancing therapies.

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10. Standard activation as iminoyl-chloride resulted in low yield reactions due to instability during the necessary aqueous work-up. The corresponding iminoyl-triazolide was in contrast stable and convenient to handle.
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