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Tricyclic isoxazolines: Identification of R226161 as a potential new antidepressant that combines potent serotonin reuptake inhibition and α_2-adrenoceptor antagonism

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Abstract—In previous articles we have described the discovery of a new series of tricyclic isoxazolines combining central serotonin (5-HT) reuptake inhibition with α_2-adrenoceptor antagonistic activity. We report now on the synthesis, the in vitro binding potency and the primary in vivo activity of six enantiomers within this series, one of which was selected for further pharmacological evaluation and assigned as R226161. Some additional in vivo studies in rats are described with this compound, which proved to be centrally and orally active as a combined 5-HT reuptake inhibitor and α_2-adrenoceptor antagonist.

1. Introduction

Major depression is a serious and incapacitating disorder with a heavy social burden and a substantial lifetime risk, which is among the most prevalent forms of mental illness and affects an estimated 18 million people in the US and 350 million worldwide.[1,2] It is common in the elderly and is increasingly recognized in young adults, adolescents and children. In the United States alone, it is currently estimated that more than 21% of women and 13% of men will suffer major depressive disorder (MDD) at some point in their lifetime, and the resulting cost to the US economy is in the range of US $100 billion per year.[2–4] By 2020, MDD is expected to be the second leading cause of disease or injury both in developing and developed countries.[1] The monoaminergic hypothesis of depression assumes that depression is caused by the dysfunction of the serotonin (5-HT, SER), noradrenaline (NE) and/or dopamine (DA) neurotransmitter systems.[5] This hypothesis has been used to explain the efficacy of existing antidepressant therapies. Among these available therapies, selective serotonin reuptake inhibitors (SSRIs) and more recently combined serotonin- and noradrenaline reuptake inhibitors (SNRIs) have become the standard treatment for depression. These agents have a less serious side-effect profile than first-generation drugs, but are neither more efficacious nor more rapidly acting than them. Although the large majority of depressed individuals (~70%) exhibit some improvement with antidepressant medication, only ~50% or even less of all patients exhibit remission.[6] Moreover, SSRIs and SNRIs require 2–4 weeks for manifestation of significant therapeutic effects and show other limitations, such as inducing nausea as well as sexual dysfunction.[7]

Keywords: Isoxazolines; α_2-Adrenoceptor blockade; Serotonin uptake inhibition; Antidepressant.
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α_2-Adrenoceptors play an important role in the regulation of neurotransmitter release.[8] First of all, release-regulating α_2-adrenoceptors are present on the terminals of noradrenergic neurons. NE released into the synaptic cleft is thought to stimulate these presynaptic autoreceptors, triggering a negative feedback mechanism that acts to inhibit subsequent neurotransmitter release. In addition to their presynaptic location, α_2-autoreceptors are found on the cell bodies of locus coeruleus neurons. Antagonizing α_2-autoreceptors effectively disinhibits noradrenergic neurons and increases the production of NE. In addition, α_2 antagonists disinhibit 5-HT neurons directly via α_2 antagonism of heteroreceptors and indirectly via increased NE input, thereby increasing 5-HT release in the dorsal raphe nucleus. The combined actions of α_2 antagonism result in the dual enhancement of 5-HT and NE release, which could produce a more rapid and/or enhanced antidepressant effect.[9,10] Several preliminary clinical studies appear to support this premise by demonstrating effective augmentation of SSRI medications with non-selective α_2-adrenoceptor antagonists such as mirtazapine or yohimbine.[11] The support for this approach is endorsed by more recent clinical findings showing that such combination therapies help with treatment-resistant depression and can actually shorten the time required to achieve antidepressant activity.[12,13] In addition, α_2-adrenoceptor antagonism improves sexual function as shown by treatment with the α_2-adrenoceptor antagonist yohimbine,[11] thereby potentially reducing one of the side effects related to 5-HT reuptake inhibition.[14] All these precedents suggest that combining 5-HT reuptake inhibition with α_2-adrenoceptor antagonism, either in a single molecule or as an adjunctive therapy, could have a therapeutic utility in the treatment of MDD.

In recent years only a few other groups have reported compounds that combine 5-HT reuptake inhibition and α_2-adrenoceptor blockade: Sterling-Winthrop's napamezole,[15] Abbott's A-80426[16] or Servier's S-34324[17] (Chart 1). In our previous papers we have described the discovery of a new series of tricyclic isoxazolines **I** (Chart 1) displaying this dual activity.[18–21]

Amongst the most interesting compounds within that series the 3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole derivatives, ***cis*-1** and ***cis*-2**, and the 3,3a,4,5-tetrahydroquinolino[4,3-*c*]isoxazole derivative ***cis*-3** were identified (Chart 2). We have reported that while the affinity at the 5-HT transporter (5-HTT) site was comparable for each pair of enantiomers of compounds ***cis*-1** and ***cis*-2**, the corresponding (+)-enantiomer proved to be more active for the α_2-adrenoceptors than the (−)-enantiomer.[20] In the case of the tetrahydroquinolino[4,3-*c*]isoxazole analogues, we identified derivative ***cis*-3** as the most active compound in our in vivo assay predictive for central $\alpha_2$blockade, inhibition of medetomidine-induced loss of righting,[20] but we did not report the pharmacological activity of its corresponding isolated enantiomers. We have also shown that the introduction of a fluorine atom on the phenyl ring of either the cinnamyl or methylcinnamyl moieties in the benzopyrano series somewhat improved or at least retained the activity for both 5-HTT and α_2-adrenoceptors.[19] In this article, we report on the synthesis and primary pharmacological activity of the three pairs of enantiomers of compounds ***cis*-3**, ***cis*-4** and ***cis*-5**, as well as further biological evaluation of ***cis*-(+)-5** (assigned R226161) that proved to be centrally and orally active for both the 5-HTT site and the α_2-adrenoceptors.

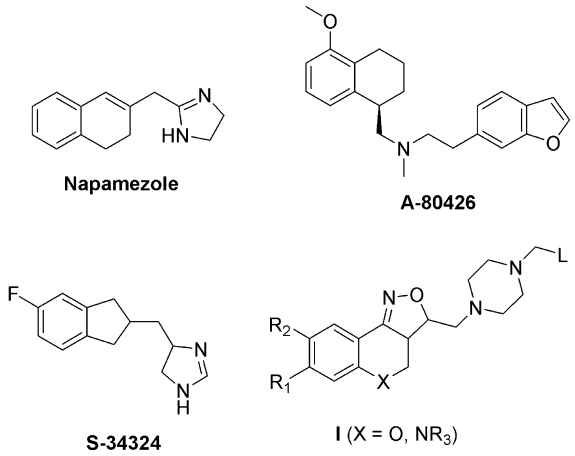


Chart 1. Combined 5-HTT inhibitors and α_2 antagonists.

2. Chemistry

The synthesis of the final *cis*-3-piperazinylmethyl-3a,4-dihydro-3*H*-[1]benzopyrano[4,3]-isoxazole derivatives ***cis*-4** and ***cis*-5** is despicted in Scheme 1. Mesylate ***cis*-(±)-6**, whose synthesis was described in our previous article,[20] was separated into its enantiomers ***cis*-(+)-6** and ***cis*-(−)-6** by chiral HPLC. Reaction of these mesylates with excess of *N*-*tert*-butyloxycarbonylpiperazine afforded the *N*-Boc-protected intermediates ***cis*-(+)-7** and ***cis*-(−)-7** that were subsequently deprotected with trifluoroacetic acid, to provide the piperazinyl derivatives ***cis*-(+)-8** and ***cis*-(−)-8**, respectively. Finally, reductive amination with the required aldehydes *trans*-4-fluoro-cinnamaldehyde[22] and 3-(4-fluorophenyl)-2-methyl-2-propenal[23] in the presence of sodium triacetoxyborohydride afforded the desired final compounds ***cis*-(+)-4**, ***cis*-(−)-4**, ***cis*-(+)-5** and ***cis*-(−)-5**. The relative *cis* configuration between the 3-exocyclic chain and the 3a-hydrogen atom was kept unaltered during the different synthesis steps, as proven by NMR. The two enantiomers ***cis*-(+)-3** and ***cis*-(−)-3** were isolated by preparative HPLC from racemic *cis*-7,8-dimethoxy-3-[4-(2-methyl-3-phenyl-2(*E*)-propen-1-yl)piperazin-1-yl-methyl]-3,3a,4,5-tetrahydroquinolino[4,3-*c*]isoxazole ***cis*-(±)-3**, prepared following our previously reported procedure.[20] Attempts to elucidate the absolute configuration of compound ***cis*-(+)-5** are currently ongoing.

3. Pharmacological results and discussion

3.1. Activity of the pairs of enantiomers *cis*-(−)-3-*cis*-(−)-5 and *cis*-(+)-3-*cis*-(+)-5

The binding affinities of the tricyclic isoxazolines for the α_2-adrenoceptors and the 5-HTT site are shown in Table 1. As we could expect from our previously published

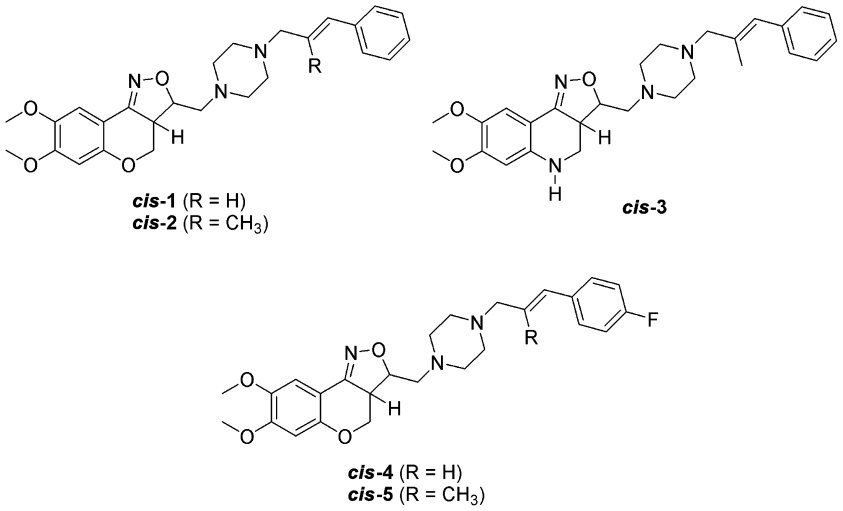
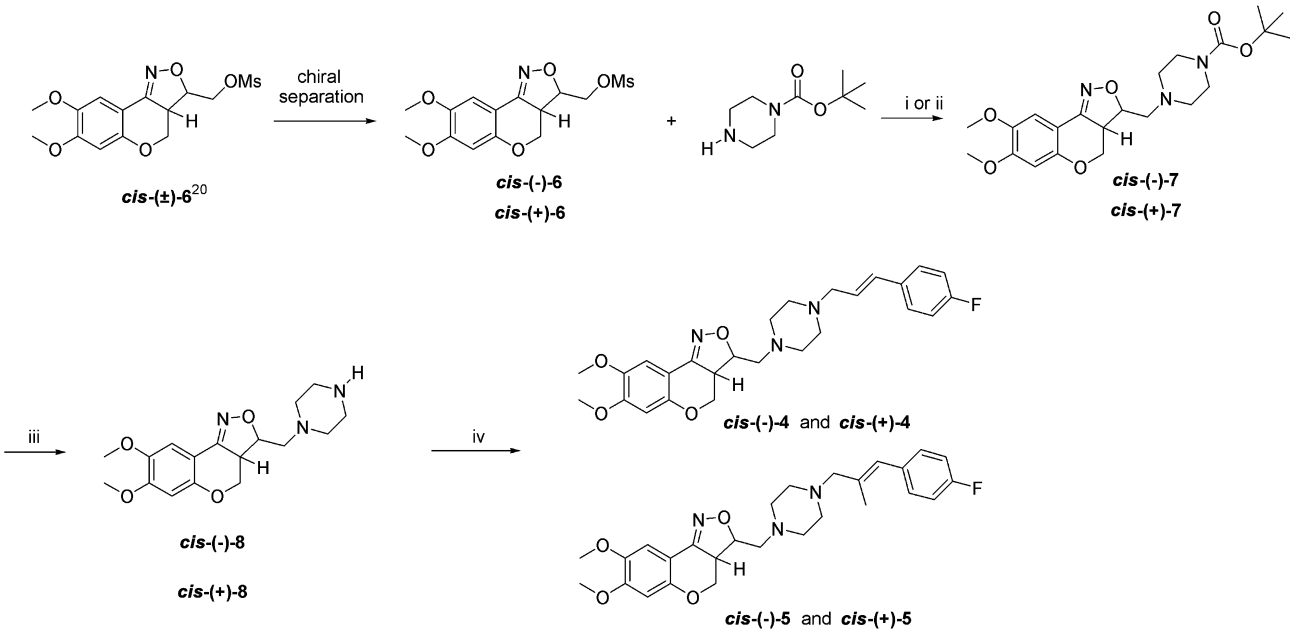


Chart 2. Structures of selected tetracyclic isoxazolines.

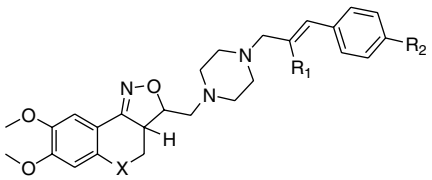


Scheme 1. Synthesis of 3-piperazinylmethyl-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole derivatives. Reagents and conditions: (i) KI, K_2CO_3, MIK, reflux, overnight; (ii) dioxane, reflux, 48 h; (iii) CF_3COOH, CH_2Cl_2, rt, 1–3 h; (iv) **L**-CHO, $NaBH(AcO)_3$, CH_3COOH (cat.), $ClCH_2CH_2Cl$, rt, 2.5–18 h.

results, the benzopyrano[4,3-*c*]isoxazole enantiomers ***cis***-(+)-**4** and ***cis***-(+)-**5** exhibited significantly higher affinity for the α_2-adrenoceptors than their corresponding pairs—***cis***-(−)-**4** and ***cis***-(−)-**5**. In addition, compound ***cis***-(+)-**5** showed activity in the nanomolar and subnanomolar range and proved to be 10- to 17-fold more potent than ***cis***-(+)-**4** at the three different α_2-adrenoceptor subtypes. Regarding their binding affinity at the 5-HTT site, both compounds showed comparable potency ($K_i \sim 2$ nM) but interestingly, while compound ***cis***-(+)-**4** was almost 7-fold more potent than its counterpart ***cis***-(−)-**4**, compound ***cis***-(−)-**5** showed nearly the same affinity as enantiomer ***cis***-(+)-**5** ($K_i = 3.7$ and 1.7 nM, respectively). In contrast, the tetrahydroquinolino[4,3-*c*]isoxazole enantiomers, that is, ***cis***-(+)-**3** and ***cis***-(−)-**3**, exhibited differential in vitro profiles. Thus, compound ***cis***-(+)-**3** showed nanomolar affinity for the α_2-adrenoceptors but it exhibited marked reduction in activity at the 5-HTT site ($K_i = 79$ nM), while compound ***cis***-(−)-**3** showed much less affinity for the three α_2-adrenoceptor subtypes, specially the α_{2A}, and 3-fold higher potency for 5-HTT relative to ***cis***-(+)-**3**. We have observed a similar dissociation of α_2 blockade and 5-HTT inhibitory activity in a pair of enantiomers of our previously published *cis*-7-amino-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole derivatives.[21]

This dissociation was also confirmed in our in vivo assays: (1) inhibition of *p*-chloroamphetamine (pCA)-induced excitation, which evaluates the ability of compounds to block the central 5-HTT, and (2) inhibition of medetomidine-induced loss of righting, which

Table 1. In vitro binding affinities of the selected tetracyclic isoxazolines



Compound	X	R_1	R_2	K_i[a] (nM)			
				α_{2A}	α_{2B}	α_{2C}	5-HTT
***cis*-(±)-3**[20]	NH	CH_3	H	2.4	12	0.1	8.9
***cis*-(−)-3**	NH	CH_3	H	87	26	8.3	24
***cis*-(+)-3**	NH	CH_3	H	1.3	4.5	0.93	79
***cis*-(±)-4**[19]	O	H	F	9.0	25	23	5.6
***cis*-(−)-4**	O	H	F	117	162	72	14
***cis*-(+)-4**	O	H	F	54	14	4.5	2.1
***cis*-(±)-5**[19]	O	CH_3	F	3.4	8.5	0.7	1.2
***cis*-(−)-5**	O	CH_3	F	28	21	10	3.7
***cis*-(+)-5**	O	CH_3	F	3.1	1.5	0.27	1.7

[a] K_i values shown are obtained from at least two confirmatory experiments differing by less than 0.5 log units in pIC_{50}. The K_i values represent the concentration giving half-maximal inhibition of [^{3}H]rauwolscine (α_{2A}, α_{2B} and α_{2C}) and [^{3}H]paroxetine (5-HTT) binding to cloned human receptors.

Table 2. ED_{50} values of the selected tetracyclic isoxazolines for reversal of medetomidine-induced loss of righting, antagonism of pCA-induced excitation and pCA-induced 5-HT depletion in vivo, 1 h after subcutaneous (sc) and oral (po) administration

Compound	ED_{50}[a] (mg/kg)					
	Medetomidine (sc)	Medetomidine (po)	pCA (sc)	pCA (po)	5-HT depletion (sc)	5-HT depletion (po)
***cis*-(±)-3**[20]	0.20 (0.13-0.29)	nt	2.2 (1.2-4.0)	nt	nt	nt
***cis*-(−)-3**	>10	>10	0.32 (0.16-0.63)	3.2 (1.4-6.9)	0.81 (0.30-2.5)	nt
***cis*-(+)-3**	0.08 (0.04-0.16)	0.63	>10	>10	4.1 (3.1-5.2)	nt
***cis*-(±)-4**[19]	0.1 (0.06-0.3)	nt	4.7 (1.7-8.5)	nt	nt	nt
***cis*-(−)-4**	nt	nt	nt	nt	nt	nt
***cis*-(+)-4**	3.1 (2.3-4.2)	3.5 (2.4-5.3)	0.05 (0.02-0.10)	0.32 (0.16-0.63)	0.03 (0.01-0.05)	0.05 (0.02-0.10)
***cis*-(±)-5**[19]	0.1 (0.04-0.16)	nt	10	nt	nt	nt
***cis*-(−)-5**	5.0 (2.5-9.9)	nt	3.2 (1.1-9.0)	nt	nt	nt
***cis*-(+)-5**	2.7 (2.0-3.6)	1.3 (0.90-2.0)	0.074 (0.046-0.12)	0.77 (0.52-1.2)	0.04 (0.02-0.06)	0.15 (0.11-0.20)

[a] 95% confidence limits are shown in parentheses. All values are mean values for $n = 3$–5/dose. nt, not tested.

evaluates the ability to block central α_2-adrenoceptors.[20] Some of the compounds were also evaluated against pCA-induced 5-HT depletion (Table 2). Compound ***cis*-(−)-3**was not active at a dose of 10 mg/kg in the medetomidine test, as it could be expected from its binding data for α_2-adrenoceptors, but was quite potent in both pCA assays in good correlation with its 5-HTT affinity. On the other hand, enantiomer ***cis*-(+)-3** proved to be very potent in the medetomidine test, both subcutaneously and orally, in accordance to its affinity for the α_2-adrenoceptors, but was inactive in the pCA-induced excitation assay although it also exhibited some weak activity in the pCA-induced 5-HT depletion test. The two benzopyrano[4,3-*c*]isoxazole enantiomers ***cis*-(+)-4** and ***cis*-(+)-5** were active in the medetomidine and in the pCA assays. In the medetomidine test, the methylcinnamyl derivative ***cis*-(+)-5** and the cinnamyl analogue ***cis*-(+)-4** showed quite comparable potencies, both after subcutaneous and after oral administration. This similarity in in vivo potency did not correspond to their respective binding affinity values for α_2-adrenoceptors, as we might have predicted a more marked reduction in the activity of the latter compound in view of its in vitro data. It is noteworthy that compound ***cis*-(+)-5** showed similar potency after both types of administration in this medetomidine test. The activities of both compounds in the pCA assays were comparable and in good correlation with their respective binding affinities for the 5-HTT site. Compound ***cis*-(−)-5** was tested subcutaneously in the medetomidine and in the pCA-induced excitation assays, showing a marked reduction in activity relative to its counterpart ***cis*-(+)-5**, specially in the pCA test, and a nice correlation with the binding data. We did not consider of interest to evaluate the in vivo activity of analogue ***cis*-(−)-4**, due to its lower affinity for the α_2-adrenoceptors. The potent central and oral activity as 5-HT reuptake inhibitor and as α_2-adrenoceptor antagonist of the methylcinnamyl derivative ***cis*-(+)-5** (R226161) prompted us to select it for further pharmacological studies.

3.2. Further pharmacological evaluation of *cis*-(+)-5 (R226161)

The binding affinity of R226161 was evaluated for a panel of more than 50 neurotransmitter receptors and transporters as well as ion channels. The compound showed only weak to moderate affinity for the H_1

Table 3. In vitro binding affinities of R226161 for various receptors and transporter sites

Compound	K_i[a] (nM)									
	α_{2A}	α_{2B}	α_{2C}	5-HTT	α_{1A}	H_1	D_3	NK_1	DAT	NET
R226161	3.1	1.5	0.27	1.7	125	74	375	310	583	70

[a] The activity of compounds was confirmed in an independent experiment. A difference in pIC_{50} up to 0.6 (S.D. < 0.5) was considered as reproducible and therefore accepted. The K_i values represent the concentration giving half-maximal inhibition of [^{3}H]rauwolscine (α_{2A}, α_{2B} and α_{2C}), [^{3}H]paroxetine (5-HTT), [^{3}H]prazosin (α_1), [^{3}H]pyrilamine (H_1), [^{125}I]iodosulpride (D_3), [^{3}H]substance P (NK1), [^{3}H]WIN35428 (DAT) and [^{3}H]nisoxetine (NET) binding to cloned human receptors or (for DAT and NET) to rat tissue. Binding affinity was evaluated on >50 neurotransmitter receptors and transporters and ion channels. For all others tested affinity values were K_i > 1000 nM.

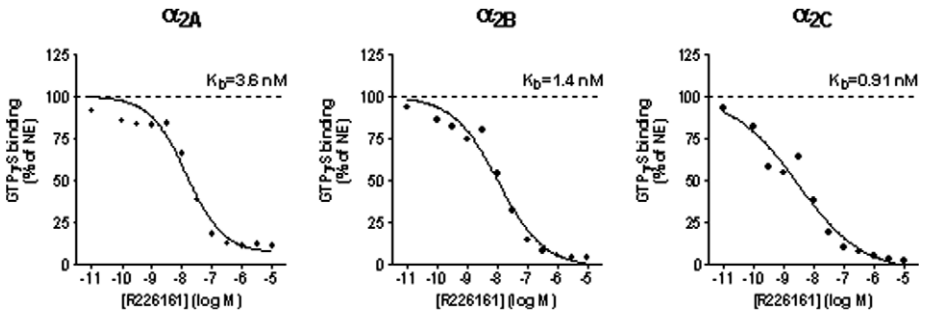


Figure 1. Functional potency of R226161 for human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors measured in a [^{35}S]GTPgS binding experiment.

receptor, NE transporter and α_{1A}-adrenoceptor (Table 3). For all other targets tested the affinity was >100-fold lower than for the 5-HTT, including the hERG ion channel in which the compound, tested up to 10^{-4} M, did not interact with the [^{3}H]astemizole binding site.

Our selected compound, tested up to 10^{-5} M, did not exert any agonistic activity on hα_{2A}-, hα_{2B}- and hα_{2C}-adrenoceptors as measured by [^{35}S]GTPγS binding. On the contrary, it proved to be a full antagonist on the three human α_2-adrenoceptor subtypes, evaluated by [^{35}S]GTPγS binding: α_{2A}, K_b = 3.6 nM; α_{2B}, K_b = 1.4 nM and α_{2C}, K_b = 0.91 nM (Fig. 1).

Occupancy of the 5-HTT and α_2-adrenoceptors was measured in the frontal cortex and the entorhinal cortex, respectively, using ex vivo autoradiography methods. One hour after subcutaneous injection, R226161 potently occupied central 5-HTT (ED_{50} = 0.10 mg/kg) and to a lesser extent α_2-adrenoceptors (ED_{50} = 2.60 mg/kg). After oral administration the compound was found to be 4–6 times less potent (ED_{50} = 0.60 and 8.8 mg/kg, respectively) (Table 4).

After determination of ED_{50}-values, a time course of 5-HTT and α_2-adrenoceptors' occupancy by R226161 was also performed. This experiment showed that the maximal occupancy of 5-HTT and α_2-adrenoceptors by R226161 was reached 1–2 h after by either route of administration (Fig. 2). This experiment indicates also that our selected compound at the doses used would be active in rats for at least 8 h, since relevant levels of 5-HTT occupancy could still be observed at this time point.

Table 4. Ex vivo occupancy of 5-HTT and α_2-adrenoceptors by R226161, 1 h after subcutaneous and oral administration

Target	Subcutaneous, ED_{50}[a] (mg/kg)	Oral, ED_{50}[a] (mg/kg)
5-HTT	0.10 (0.08–0.12)	0.60 (0.49–0.73)
α_2	2.6 (1.7–3.8)	8.8 (6.4–12)

[a] 95% confidence limits are shown in parentheses. All values are mean values for n = 3/dose.

Once the ED_{50}-values of R226161 in the pCA-induced excitation and the medetomidine tests were determined, one hour after subcutaneous and oral administration as shown in Table 2, a time course for both assays was also performed. The compound dose-dependently reversed pCA-induced excitation in the absence of overt sedative effects. The ED_{50}'s obtained at several time intervals after subcutaneous or oral administration are listed in Table 5. Plotting these ED_{50}'s versus time (Fig. 3), graphical estimation revealed a peak-effect dose of 0.10 mg/kg, an onset of less than 30 min., and a duration of action of 15 h (at four times the peak-effect dose) after subcutaneous injection and a peak-effect dose of 0.32 mg/kg, an onset of less than 30 min, and a duration of action of 21 h (at four times the peak-effect dose) after oral administration.

R226161 dose-dependently reversed medetomidine-induced loss of righting at several time intervals after subcutaneous or oral administration as well (Table 6). Plotting these ED_{50}'s versus time (Fig. 3), graphical estimation revealed a peak-effect dose of 2.0 mg/kg, an onset of less than 30 min, and a duration of action of 14 h (at four times the peak-effect dose) after subcutaneous injection and a peak-effect dose of 1.0 mg/kg, an onset of less than 30 min, and a duration of action of 3.7 h (at four times the peak-effect dose) after oral administration.

In summary, our compound showed more potent and central activity in rats as a 5-HT uptake inhibitor than

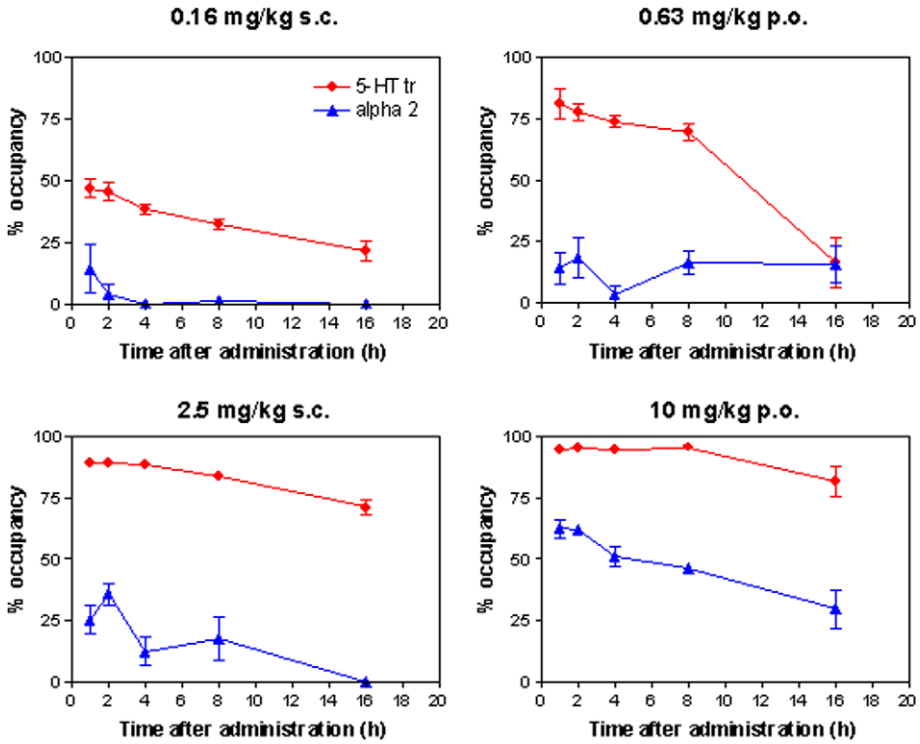


Figure 2. Time course of in vivo occupancy of 5-HTT and α_2-adrenoceptors by R226161 after subcutaneous and oral administration. All values are mean values for $n = 3$/dose.

Table 5. ED_{50} values of R226161 for inhibition of pCA-induced excitation in rats obtained at the indicated time interval after subcutaneous or oral administration

Time (h)	ED_{50}[a] (mg/kg, sc)	
	sc route	po route
0.5	0.17 (0.10–0.27)	0.67 (0.42–1.1)
1	0.074 (0.046–0.12)	0.77 (0.52–1.2)
2	0.086 (0.063–0.12)	0.26 (0.19–0.35)
4	0.26 (0.19–0.35)	0.39 (0.22–0.66)
8	0.17 (0.12–0.23)	0.58 (0.43–0.79)
16	0.26 (0.17–0.39)	0.89 (0.59–1.3)
32	1.0 (0.63–1.6)	3.1 (1.9–5.0)

[a] 95% confidence limits are shown in parentheses. All values are mean values for $n = 5$/dose.

as an α_2-adrenoceptor antagonist. Figure 3 shows the comparison between the activity of the compound in the medetomidine-induced loss of righting and the pCA-induced excitation tests after subcutaneous or oral administration. We have not found yet a consistent explanation for the relatively short duration of action of the compound after oral administration in the medetomidine test when compared with the duration after oral administration in the pCA assay. The major metabolite in rats of R226161 was found to be the nor-compound ***cis*-(+)-8** originated from the loss of the cinnamyl fragment.[24] Its binding affinity for the 5-HTT site and the α_2-adrenoceptors was evaluated and proved to be negligible ($K_i > 1000$ nM). Therefore the difference in duration of action could not be explained by the formation of a main metabolite that would show more potent affinity at 5-HTT than at α_2-adrenoceptors. Further PK/PD studies are currently ongoing trying to confirm and to explain the reasons for this finding.

4. Conclusions

Following our research programme on the tricyclic isoxazolines, which combine 5-HT reuptake inhibition and α_2-adrenoceptor antagonism, we have evaluated the in vitro binding potency and the primary in vivo activity of the four enantiomers of a pair of racemic 3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole derivatives and the two enantiomers of one racemic 3,3a,4,5-tetrahydroquinolino[4,3-*c*]isoxazole derivative, which we had previously described.[19,20] From the available data we decided to select the 3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole derivative ***cis*-(+)-5**, which was assigned as **R226161**, for further pharmacological evaluation. The compound proved to be a full antagonist on the three human α_2-adrenoceptor subtypes. In addition, R226161 potently occupied central 5-HTT sites and to a lesser extent α_2-adrenoceptors, both after subcutaneous and oral administration. A time course study for the pCA-induced excitation and the medetomidine tests was performed with R226161. Our selected compound showed more potent central activity in rats and longer duration of action as a 5-HT uptake inhibitor than as an α_2-adrenoceptor antagonist. Further in vivo studies are currently ongoing, such as full pharmacokinetic profiling in different animal species, evaluation in a set of animal paradigms predictive for

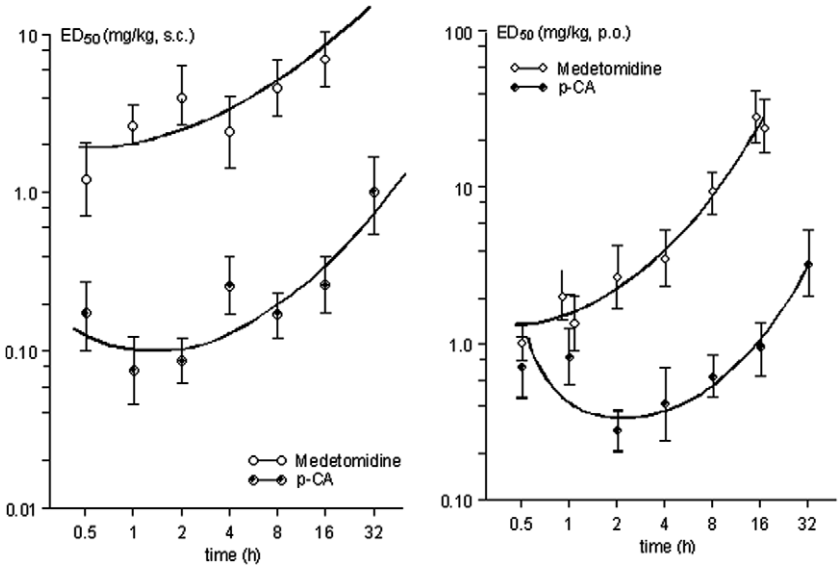


Figure 3. Comparison of the time-activity course in the pCA (closed symbols) and medetomidine (open symbols) tests after sc injection and po administration of R226161. All values are mean values for $n = 5$/dose.

Table 6. ED_{50} values of R226161 for reversal of medetomidine-induced loss of righting in rats obtained at the indicated time interval after subcutaneous or oral administration

Time interval (h)	ED_{50}[a] (mg/kg)	
	sc route	po route
0.5	1.2 (0.68–2.0)	1.0 (0.75–1.4)
1	2.7 (2.0–3.6)	1.3 (0.90–2.0)
2	4.1 (2.7–6.1)	2.7 (1.7–4.3)
4	2.3 (1.4–3.8)	3.6 (2.4–5.3)
8	4.7 (3.1–7.0)	9.3 (6.9–13)
16	7.1 (4.7–11)	25 (16–40)
32	>10	

[a] 95% confidence limits are shown in parentheses. All values are mean values for $n = 5$/dose.

antidepressant and antianxiety activity as well as toxicological and safety studies, to fully evaluate our selected compound R226161 as a potential candidate for clinical trials as an antidepressant agent. The results of these more advanced studies will be reported elsewhere.

5. Experimental

5.1. Chemistry

Melting points were determined in open capillary tubes on a Mettler FP62 apparatus and are uncorrected. Elemental analyses are within ±0.4% of the theoretical values. Chiral preparative chromatography was performed on a Waters Delta Prep 4000 with a 5 cm i.d. Prochrom D.A.C. column. The enantiomeric excess was determined by HPLC using a Waters Alliance 2690 instrument with chiral columns (Chiralcel OD, Chiralcel OJ, Chiralpak AD and Chiralpak AS, Daicel 10 μm). Optical rotations were measured on a Perkin-Elmer 341 polarimeter with a sodium lamp and reported as follows: $[\alpha]_{\lambda}^{t^{\circ}C}$ (*c* g/100 ml, solvent). ^{1}H NMR spectra were recorded on a Bruker DPX-400 with standard pulse sequences, operating at 400 MHz. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS), which was used as internal standard. HPLC-MS analysis was done with an Agilent Technologies 1100 series consisting of a quaternary pump with degasser, autosampler, column oven (set at 60 °C) and DAD detector. Flow from the column was split to the MS detector. A generic gradient: 80/10/10 $AcONH_4$ 0.05%/MeOH/CH_3CN to 50:50 CH_3CN/MeOH in 6.5 min to 100% CH_3CN at 7 min and equilibrated to initial conditions at 7.5 min until 9.0 min was performed on an ACE C-18 30 × 4.6 mm i.d. 3.0 μm column from Advanced Chromatography Technologies, with a flow rate of 1.5 ml/min. High-resolution mass spectra were recorded on a Micromass LCT Time of Flight mass spectrometer configured with an electrospray ionization source, maintained at 140 °C, using nitrogen as the nebulizer gas and Lockmass device for mass calibration using Leucine-Enkephaline as standard substance. Spectra were acquired in positive ionization mode by scanning from 100 to 750 in 0.5 s using a dwell time of 0.1 s. The capillary needle voltage was 2.5 kV and the cone voltage was 20 V. Data acquisition was performed with MassLynx-Openlynx software. Thin layer chromatography (TLC) was carried out on silica gel 60 F_{254} plates (Merck) using reagent grade solvents. Flash column chromatography was performed on silica gel, particle size 60 , mesh = 230–400 (Merck).

5.1.1. Resolution of racemic mixture of *cis*-(±)-methanesulfonic acid 7,8-dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazol-3-ylmethyl ester (*cis*-(±)-6). *cis*-Methanesulfonic acid 7,8-dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazol-3-ylmethyl ester ***cis*-(±)-6** (200 g, 0.58 mol) was separated into its enantiomers by preparative chiral HPLC over LC110-2 column with Chiralpak-AD as stationary phase (methanol/acetonitrile 70:30 v/v). Two pure fraction groups were collected

and their solvents were evaporated, to yield 105 g of compound ***cis*-(−)-6** as a white solid: mp 187.6 °C; 99%ee; $[\alpha]_D^{20}$ −142.00° (*c* 0.13, DMF); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 3.27 (s, 3H) 3.73 (s, 3H) 3.75–3.82 (m, 1H) 3.78 (s, 3H) 4.13 (dd, J = 12.44, 10.57 Hz, 1H) 4.48–4.61 (m, 2H) 4.62–4.76 (m, 2H) 6.61 (s, 1H) 7.03 (s, 1H). HRMS Calcd for $C_{14}H_{18}NO_7S$ (M+1): 344.0804. Found: 344.0792; and 95 g of compound ***cis*-(+)-6** as a white solid: mp 184.9 °C; 99%ee; $[\alpha]_D^{20}$ +152.70° (*c* 0.13, DMF); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 3.27 (s, 3H) 3.73 (s, 3H) 3.74–3.82 (m, 1H) 3.78 (s, 3H) 4.13 (dd, J = 12.44, 10.57 Hz, 1H) 4.48–4.61 (m, 2H) 4.62–4.74 (m, 2H) 6.61 (s, 1H) 7.02 (s, 1H). HRMS Calcd for $C_{14}H_{18}NO_7S$ (M+1): 344.0804. Found: 344.0801.

5.1.2. *cis*-(−)-4-(7,8-Dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4, 3-*c*]isoxazol-3-ylmethyl)piperazine-1-carboxylic acid *tert*-butyl ester (*cis*-(−)-7). A mixture of *cis*-(−)-methanesulfonic acid 7,8-dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazol-3-ylmethyl ester ***cis*-(−)-6** (15 g, 43.6 mmol), 1-*tert*-butoxycarbonylpiperazine (16.2 g, 87.3 mmol), KI (7.2 g, 43.6 mmol) and K_2CO_3 (6 g, 43.6 mmol) in methylisobutylketone (300 mL) was stirred at reflux temperature overnight. After addition of water the mixture was extracted with dichloromethane. The organic layer was dried (Na_2SO_4), filtered off and the solvent was evaporated. The residue was purified by column chromatography over silica gel (dichloromethane/methanol 97:3). The desired fractions were collected and the solvent was evaporated to give 18.9 g of ***cis*-(−)-7** as an oil, which was used for next step without further purification.

5.1.3. *cis*-(−)-7,8-Dimethoxy-3-piperazin-1-ylmethyl-3a, 4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole (*cis*-(−)-8). Trifluoroacetic acid (96 ml) was added dropwise to a solution of *cis*-(−)-4-(7,8-dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazol-3-ylmethyl)piperazine-1-carboxylic acid *tert*-butyl ester ***cis*-(−)-7** (18.9 g, 43.6 mmol) obtained in the previous step in dichloromethane (300 ml). The reaction mixture was stirred for 3 h at room temperature. The solvent was evaporated and the residue was basified with a 50% aqueous NaOH solution and extracted with dichloromethane. The organic layer was dried (Na_2SO_4), filtered and the solvent was evaporated to yield 14.4 g (99%, two steps) of ***cis*-(−)-8** as a white foam: 99%ee; $[\alpha]_D^{20}$ −110.60° (*c* 0.13, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.40–2.71 (br s, 4H) 2.76–2.91 (m, 2H) 2.81–2.99 (m, 4H) 3.67 (td, J = 12.54, 5.80 Hz, 1H) 3.86 (s, 3H) 3.88 (s, 3H) 4.07 (dd, J = 12.63, 10.48 Hz, 1H) 4.41 (ddd, J = 12.24, 6.01, 5.81 Hz, 1H) 4.59 (dd, J = 10.47, 5.80 Hz, 1H) 6.46 (s, 1H) 7.17 (s, 1H). HRMS Calcd for $C_{17}H_{24}N_3O_4$ (M+1): 344.1767. Found: 344.1760.

5.1.4. *cis*-(+)-4-(7,8-Dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazol-3-ylmethyl)piperazine-1-carboxylic acid *tert*-butyl ester (*cis*-(+)-7). A mixture of *cis*-(+)-methanesulfonic acid 7,8-dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazol-3- ylmethyl ester ***cis*-(+)-6** (25 g, 72.8 mmol) and 1-*tert*-butoxycarbonylpiperazine (16 g, 87 mmol) in dioxane (500 ml) was stirred and refluxed for 48 h. The solvent was evaporated and dichloromethane was added first, then water and 50% aqueous NaOH solution were added also and the mixture was extracted with dichloromethane. The separated organic layer was dried (Na_2SO_4), filtered and the solvent was evaporated under vacuum to yield 32 g of compound ***cis*-(+)-7** as an oil, which was used for next step without further purification.

5.1.5. *cis*-(+)-7,8-Dimethoxy-3-piperazin-1-ylmethyl-3a, 4-dihydro- 3*H*-[1]benzopyrano[4,3-*c*]isoxazole (*cis*-(+)-8). A mixture of *cis*-(+)-4-(7,8-dimethoxy-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazol-3-ylmethyl)piperazine-1-carboxylic acid *tert*-butyl ester ***cis*-(+)-7** (32 g, 72.8 mmol) and trifluoroacetic acid (189 ml) in dichloromethane (500 ml) was stirred for 1 h at room temperature. The solvent was evaporated and the residue was dissolved in dichloromethane. Then 50% aqueous NaOH solution was added and the mixture was extracted. The separated organic layer was dried (Na_2SO_4), filtered and the solvent was evaporated. The residue was purified by column chromatography over silica gel (dichloromethane/methanol saturated with ammonia 100:0 to 95:5). The pure fractions were collected and the solvent was evaporated affording 14.32 g (59%, two steps) of compound ***cis*-(+)-8** as a white solid: mp 67.0 °C; 99%ee; $[\alpha]_D^{20}$ +114.80° (*c* 0.13, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.40–2.70 (br s, 4H) 2.75–2.90 (m, 2H) 2.82–2.99 (m, 4H) 3.67 (td, J = 12.54, 5.81 Hz, 1H) 3.86 (s, 3H) 3.88 (s, 3H) 4.07 (dd, J = 12.65, 10.37 Hz, 1H) 4.41 (ddd, J = 12.23, 6.01, 5.80 Hz, 1H) 4.59 (dd, J = 10.37, 5.80 Hz, 1H) 6.46 (s, 1H) 7.17 (s, 1H). HRMS Calcd for $C_{17}H_{24}N_3O_4$ (M+1): 344.1767. Found: 344.1755.

5.1.6. *cis*-(+)-7,8-Dimethoxy-3-[4-(3-(4-fluoro)phenyl-2(*E*)-propen-1-yl)piperazin-1-ylmethyl]-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole (*cis*-(+)-4). A mixture of *cis*-(+)-7,8-dimethoxy-3-piperazin-1-ylmethyl-3a,4-dihydro-3*H*- [1]benzopyrano[4,3-*c*]isoxazole ***cis*-(+)-8** (6.7 g, 20 mmol), *trans*-4-fluorocinnamaldehyde[23] (4.59 g, 30 mmol) and acetic acid (1 ml) in 1,2-dichloroethane (250 ml) was stirred for 30 min at room temperature. Then sodium triacetoxyborohydride (6.3 g, 30 mmol) was added and the mixture was stirred for 2.5 h. The mixture was treated with water and extracted with dichloromethane. The separated organic layer was dried (Na_2SO_4) and the solvent was evaporated. The residue was purified by column chromatography over silica gel (dichloromethane/methanol 97:3 to 95:5). The desired fractions were collected and the solvent was evaporated. The residue was crystallized from diisopropylether and dichloromethane to afford 6.0 g (63%) of ***cis*-(+)-4** as light yellow solid: mp 145.8 °C; 99%ee; $[\alpha]_D^{20}$ +95.79° (*c* 0.55, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.56 (br s, 4H) 2.64 (br s, 4H) 2.78–2.93 (m, 2H) 3.16 (d, J = 6.63 Hz, 2H) 3.67 (td, J = 12.54, 5.80 Hz, 1H) 3.86 (s, 3H) 3.87 (s, 3H) 4.06 (dd, J = 12.65, 10.37 Hz, 1H) 4.40 (ddd, J = 12.18, 5.96, 5.70 Hz, 1H) 4.58 (dd, J = 10.16, 5.81 Hz, 1H) 6.19 (dt, J = 15.76, 6.84 Hz, 1H) 6.46 (s, 1H) 6.49 (d, J = 15.96 Hz, 1H) 7.00 (t, J = 8.71 Hz, 2H) 7.16 (s, 1H) 7.34 (dd, J = 8.71, 5.39 Hz, 2H). HRMS

Calcd for $C_{26}H_{31}FN_3O_4$ (M+1): 468.2299. Found: 468.2295.

5.1.7. *cis*-(+)-7,8-Dimethoxy-3-[4-(2-methyl-3-(4-fluoro)phenyl-2(*E*)-propen-1-yl)piperazin-1-ylmethyl]-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole (*cis*-(+)-5). A mixture of *cis*-(+)-7,8-dimethoxy-3-piperazin-1-ylmethyl-3a,4-dihydro-3*H*- [1]benzopyrano[4,3-*c*]isoxazole ***cis*-(+)-8** (7.16 g, 21.2 mmol), 3-(4-fluorophenyl)-2-methyl-2-propenal[24] (4.17 g, 25.4 mmol), sodium triacetoxyborohydride (6.7 g, 31.8 mmol) and acetic acid (two drops) in 1,2-dichloroethane (150 ml) was stirred overnight at room temperature. The reaction mixture was treated with a saturated aqueous NH_4Cl solution and extracted with dichloromethane. The organic phase was dried (Na_2SO_4), filtered and the solvent was evaporated. The residue was purified by column chromatography over silica gel (dichloromethane/methanol 98:2 to 95:5). The desired fractions were collected and the solvent was evaporated. The residue was treated with diethyl ether to yield 3.39 g (53%) of compound ***cis*-(+)-5** as a white solid: mp 108.0 °C; 99%ee; $[\alpha]_D^{20}$ +89.50° (*c* 0.55, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.87 (d, J = 0.83 Hz, 3H) 2.48 (br s, 4H) 2.62 (br s, 4H) 2.78–2.94 (m, 2H) 3.00 (s, 2H) 3.67 (td, J = 12.54, 5.80 Hz, 1H) 3.86 (s, 3H) 3.88 (s, 3H) 4.07 (dd, J = 12.54, 10.26 Hz, 1 H) 4.41 (ddd, J = 12.34, 6.01, 5.70 Hz, 1H) 4.59 (dd, J = 10.16, 5.81 Hz, 1H) 6.38 (br s, 1H) 6.46 (s, 1H) 6.95–7.06 (m, 2H) 7.16 (s, 1H) 7.19–7.26 (m, 2H). HRMS Calcd for $C_{27}H_{33}FN_3O_4$ (M+1): 482.2455. Found: 482.2477.

5.1.8. *cis*-(−)-7,8-Dimethoxy-3-[4-(3-(4-fluoro)phenyl-2(*E*)-propen-1-yl)piperazin-1-ylmethyl]-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole (*cis*-(−)-4). A mixture of **cis**-(−)-7,8-dimethoxy-3-piperazin-1-ylmethyl-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole ***cis*-(−)-8** (0.73 g, 2.2 mmol), *trans*-4-fluorocinnamaldehyde[23] (0.5 g, 3.3 mmol), sodium triacetoxyborohydride (0.69 g, 3.3 mmol) and acetic acid (two drops) in 1,2-dichloroethane (20 ml) was stirred for 3 h at room temperature. The reaction mixture was treated with a saturated aqueous NH_4Cl solution and extracted with dichloromethane. The organic phase was dried (Na_2SO_4), filtered and the solvent was evaporated. The residue was purified by column chromatography over silica gel (dichloromethane/methanol 97:3 to 94:6). The desired fractions were collected and the solvent was evaporated. The residue was treated with diethyl ether yielding 0.58 g (58%) of ***cis*-(−)-4** as a white solid: mp 224.6 °C; 99%ee; $[\alpha]_D^{20}$ −82.00° (*c* 0.51, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.70 (br s, 8 H) 2.81–2.95 (m, 2H) 3.23 (d, J = 6.43 Hz, 2H) 3.67 (td, J = 12.54, 5.81 Hz, 1H) 3.86 (s, 3H) 3.88 (s, 3 H) 4.07 (dd, J = 12.65, 10.37 Hz, 1H) 4.41 (ddd, J = 12.44, 5.80, 5.39 Hz, 1H) 4.57 (dd, J = 10.26, 5.70 Hz, 1H) 6.22 (dt, J = 15.96, 6.63 Hz, 1H) 6.46 (s, 1H) 6.51 (d, J = 15.96 Hz, 1H) 7.00 (t, J = 8.71 Hz, 2H) 7.16 (s, 1H) 7.35 (dd, J = 8.71, 5.39 Hz, 2H). HRMS Calcd for $C_{26}H_{31}FN_3O_4$ (M+1): 468.2299. Found: 468.2310.

5.1.9. *cis*-(−)-7,8-Dimethoxy-3-[4-(2-methyl-3-(4-fluoro)phenyl-2(*E*)-propen-1-yl)piperazin-1-ylmethyl]-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole (*cis*-(−)-5). A mixture of *cis*-(−)-7,8-dimethoxy-3-piperazin-1-ylmethyl-3a,4-dihydro-3*H*-[1]benzopyrano[4,3-*c*]isoxazole ***cis*-(−)-8** (0.73 g, 2.2 mmol), 3-(4-fluorophenyl)-2-methyl-2-propenal[24] (0.43 g, 2.6 mmol), sodium triacetoxyborohydride (0.69 g, 3.3 mmol) and acetic acid (two drops) in 1,2-dichloroethane (20 ml) was stirred for 3 h at room temperature. The reaction mixture was treated with a saturated aqueous NH_4Cl solution and extracted with dichloromethane. The organic phase was dried (Na_2SO_4), filtered and the solvent was evaporated. The residue was purified by column chromatography over silica gel (dichloromethane/methanol 98:2) and then by HPLC over silica gel (dichloromethane/methanol saturated with ammonia 99:1). The desired fractions were collected and the solvent was evaporated. The residue was treated with diethyl ether, filtered off and dried affording 0.14 g (14%) of ***cis*-(−)-5** as a white solid: mp 212.6 °C; 99%ee; $[\alpha]_D^{20}$ −80.80° (*c* 0.59, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.87 (s, 3H) 2.47 (br s, 4H) 2.62 (br s, 4H) 2.79–2.92 (m, 2H) 3.00 (s, 2H) 3.67 (td, J = 12.54, 5.60 Hz, 1H) 3.86 (s, 3H) 3.88 (s, 3H) 4.07 (dd, J = 12.65, 10.37 Hz, 1H) 4.41 (ddd, J = 12.23, 6.01, 5.81 Hz, 1H) 4.59 (dd, J = 10.16, 5.81 Hz, 1H) 6.37 (br s, 1H) 6.46 (s, 1H) 6.96–7.05 (m, 2H) 7.17 (s, 1H) 7.19–7.25 (m, 2H). HRMS Calcd for $C_{27}H_{33}FN_3O_4$ (M+1): 482.2455. Found: 482.2451.

5.1.10. Resolution of racemic mixture of *cis*-(±)-7,8-dimethoxy-3-[4-(2-methyl-3-phenyl-2(*E*)-propen-1-yl)piperazin-1-ylmethyl]-3,3a,4,5-tetrahydroquinolino[4,3-*c*]isoxazole (*cis*-(±)-3). Compound *cis*-(±)-7,8-dimethoxy-3-[4-(2-methyl-3-phenyl-2(*E*)-propen-1- yl)piperazin-1-ylmethyl]-3,3a,4,5-tetrahydroquinolino[4,3-*c*]isoxazole ***cis*-(±)-3**[20] (2.8 g, 6.05 mmol) was separated into its enantiomers by preparative chiral HPLC over RP BDS C18 column with Chiralcel OJ as stationary phase (0.5% ammonium acetate in water/acetonitrile 90:10/methanol 70:30). Two pure fractions were collected and the organic solvent was evaporated. In each case the aqueous layer was extracted with dichloromethane, the separated organic layer was dried ($MgSO_4$), filtered and the solvent was evaporated. The residues were stirred in hexane and the precipitates were filtered off to yield ***cis*-(+)-3** (0.69 g) as a light brown solid: mp 163.6 °C; 99%ee; $[\alpha]_D^{20}$ +18.30° (*c* 0.13, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.90 (d, J = 0.83 Hz, 3H) 2.48 (br s, 4H) 2.59 (br s, 2H) 2.64 (br s, 2H) 2.78–2.90 (m, 2H) 3.01 (s, 2H) 3.27–3.38 (m, 1H) 3.50–3.64 (m, 2H) 3.82 (s, 3H) 3.84 (s, 3H) 3.91 (br s, 1H) 4.39–4.51 (m, 1H) 6.14 (s, 1H) 6.42 (br s, 1H) 7.15 (s, 1H) 7.20 (t, J = 7.15 Hz, 1H) 7.27 (d, J = 7.05 Hz, 2H) 7.30–7.36 (m, 2H). HRMS Calcd for $C_{27}H_{35}N_4O_3$ (M+1): 463.2709. Found: 463.2715. Anal ($C_{27}H_{34}N_4O_3$) C, H, N; and ***cis*-(−)-3** (0.67 g) as a light yellow solid: mp 110.4 °C; 99%ee; $[\alpha]_D^{20}$ −17.50° (*c* 0.13, DMF); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.91 (d, J = 1.04 Hz, 3H) 2.49 (br s, 4H) 2.60 (br s, 2H) 2.65 (br s, 2H) 2.78–2.91 (m, 2H) 3.01 (s, 2H) 3.26–3.39 (m, 1H) 3.49–3.65 (m, 2H) 3.82 (s, 3H) 3.84 (s, 3H) 3.92 (br s, 1H) 4.38–4.52 (m, 1H) 6.14 (s, 1H) 6.42 (br s, 1H) 7.15 (s, 1H) 7.20 (t, J = 7.15 Hz, 1H) 7.27 (d, J = 6.84 Hz, 2H) 7.30–7.37 (m, 2H). HRMS Calcd for $C_{27}H_{35}N_4O_3$ (M+1): 463.2709. Found: 463.2713. Anal ($C_{27}H_{34}N_4O_3$) C, H, N.

5.2. Pharmacology

5.2.1. Receptor binding assays. Frozen membranes of CHO cells, stably transfected with either human adrenergic-2A, -2B or -2C receptors, were thawed on ice, briefly homogenized with an Ultra Turrax homogeniser and then suspended in glycylglycine buffer (25 mM, pH 7.6) at an appropriate pre-determined protein concentration (5–10 μg protein per incubation mixture). The reaction was started by adding the membrane suspension to the reaction tube containing the compound of interest together with [^{3}H]Rauwolscine (1 nM, final concentration) in a total volume of 500 μl. The mixture was incubated for 30 min at 25 °C. Non-specific binding was determined in the presence of oxymetazoline (1 μM, final concn) for the -2A subtype and spiroxatrine (1 μM, final concn) for -2B and -2C subtypes. Free radioligand was separated from the radioligand–receptor complex by means of rapid vacuum filtration over GF/B unifilter plates with a Packard Harvester Filtration Unit. Filter plates were washed with ice-cold Tris–HCl buffer (50 mM, pH 8.0) and dried overnight. Bound counts were measured in a Topcount Scintillation Counter in the presence of Microscint O. For 5-HTT binding, frozen membranes of human platelets (Novascreen, Maryland, USA) were thawed on ice, briefly homogenized and resuspended in Tris–HCl buffer (50 mM, pH 7.4) supplemented with NaCl (120 mM) and KCl (5 mM) at a concentration of 30–50 μg protein per incubation mixture. The membrane suspension was added to the compound of interest together with [^{3}H]paroxetine (0.5 nM, final concn) in a total volume of 250 μl and incubated (60 min, 25 °C). Non-specific binding was determined in the presence of imipramine (1 μM). Filtration was done over pre-soaked GF/B unifilters (0.1% PEI) and washed as above with the Tris salt buffer used for the incubation. Specific binding was calculated and sigmoidal curves were plotted by an internally developed software program based on S-plus software. K_i values were calculated using the Cheng-Prusoff equation.

The affinity of the compounds for the remaining target receptors and transporters was also determined by means of several radioligand competition binding experiments. In general, the compound of interest (or control blank) together with the appropriate tritiated or iodinated radioligand and a membrane suspension with abundant target receptor/transporter were incubated under optimized experimental conditions. The reaction was stopped by filtration as above, except for α_{1A} and D_3, where a SPA-based assay was used (Scintillation Proximity Assay). All assays were carried out with membranes from cell lines transfected with the human target, except for DAT and NET, which were done with the rat striatum and the rat cortex, respectively.

5.2.2. Signal transduction by [^{35}S]GTPγS assay. The [^{35}S]GTPγS binding assay was done on membranes of CHO cells permanently transfected with either human α_{2A}, α_{2B}, or α_{2C}-adrenoceptor, these cells were also used for the competition binding assay. Experimental conditions are the same for the three adrenoceptor subtypes. Noradrenaline was used to stimulate the α_2-adrenoceptors, 10 μM for maximal (agonism test) and 3 μM for submaximal stimulation (antagonism test). Frozen membranes were diluted in incubation buffer (10 mM Hepes acid, 10 mM Hepes salt, 100 mM NaCl, 1 mM $MgCl_2$ and 3 μM GDP, pH 7.4–7.5) supplemented with 10 μg/ml saponin to a concentration of 60 μg protein/ml (final 10 μg protein/assay). Assay mixtures for the agonist assay contained compound of interest or buffer (for basal levels) or 10 μM noradrenaline (for maximal levels) and 10 μg of membrane protein. For antagonism, submaximal concentrations of noradrenaline were added to compound or buffer (for submaximal levels) except for basal levels, followed by 10 μg of membrane protein. After 20 min preincubation at 37 °C, [^{35}S]GTPγS was added to a final concentration of 0.25 nM in a total volume of 250 μl. Plates were shaked for 3 s and further incubated at 37 °C for another 20 min. Reactions were terminated by rapid vacuum filtration over GF/B unifilter plates using a Packard Harvester Filtration Unit. Filters were washed three times with 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.4. Plates were dried overnight and 30 μl Microscint—O was added to each well. Membrane bound radioactivity was counted in Topcount Scintillation Counter. % Effect was calculated and curves were plotted with help of S-Plus Software. pEC_{50}/IC_{50} values were determined and K_b values were calculated for active antagonists.

5.2.3. In vivo pharmacology. *Animals*. Male Wistar rats (Charles River Breeding Facilities) were used. They were housed in individual cages in air-conditioned laboratories (21 ± 2 °C; 65 ± 15% relative humidity; 12:12 h light/dark cycle). They were fasted overnight but tap water remained available ad libitum except during the test period. Just before the experiments, the animals were transferred to individual cages.

Test compounds. Test compounds were prepared as solutions in distilled water or 10% hydroxypropyl-β-cyclodextrin after acidification with tartaric acid if necessary. They were stored at room temperature in closed containers protected from light. The solutions were subcutaneously (sc) or orally (po) administered in a volume of 10 ml/kg.

General procedure and statistics. All experiments were performed by unbiased trained technicians using coded solutions. Doses were selected from the geometrical series 0.00063-0.00125-0.0025…10-20-40 mg/kg. Animals were tested in separate daily experimental sessions in order to account for day-to-day variability and to minimize systematic errors. Control injections of solvent were included in each experimental session. All-or-none criteria for significant ($p < 0.05$) effects were defined by analyzing a frequency distribution of a large series of historical control data. On the basis of the thus obtained criteria, ED_{50} values and corresponding 95% confidence limits were determined according to the modified Spearman–Kaerber estimate using theoretical probabilities instead of empirical ones. This modification allows us to tabulate the ED_{50} and its confidence interval as a function of the slope of the log dose–response curve.[25]

Tests: medetomidine-induced loss of righting in rats. Onset and duration of medetomidine (0.10 mg/kg, iv)-induced loss of righting was recorded in overnight-starved male Wiga rats (200–250 g), pre-treated with test compound or solvent at logarithmically spaced time intervals (0.5, 1, 2, 4, 8, 16 or 32 h). Criterion for drug-induced reversal: duration = 0 min (1.0% false positive controls; $n > 400$). Criterion for drug-induced potentiation: loss of righting reflex over a period longer than 120 min (1.2% false positives). Centrally acting α_2-adrenoceptor antagonists or behavioural stimulants antagonize the loss of righting; sedative compounds may result in prolongation.

p-Chloroamphetamine (pCA)-induced behaviour in rats. *p*-Chloroamphetamine (pCA; 5 mg/10 ml/kg, sc)-induced excitation was scored (0, 1, 2, or 3) over a 15-min interval starting 45 min after the pCA injection in male rats (200–250 g) pre-treated with test compound or solvent. The following all-or-nothing criteria were selected to assess drug-induced inhibition: score for excitation <2 (0.5% false positives; $n > 200$).

5.2.4. pCA-induced 5-HT depletion assay. *Materials and methods*. To identify and characterize the in vivo central 5-HTT blockade activity, the test compound was administered subcutaneously (sc) or orally (po) (10 ml/kg) to healthy male Wistar–Wiga rats (body weight 200 ± 20 g, $n = 6$ per treatment condition). Thirty-five minutes later, pCA was administered to the animals (5 mg/kg, 1 ml/kg, ip). Four hours following pCA administration, animals were decapitated and the frontal cortex was dissected from the brain and the left and right hemisphere separated for storage. The tissue was snap-frozen in liquid nitrogen and stored at −80 °C until extraction. After a perchloric acid/sodium metabisulfite extraction, an HPLC system (LC-14AD*VP*, Shimadzu) coupled to an electrochemical detector (Procédé, Shimadzu) was used to quantify 5-HT levels (pmol/ml) in the supernatant. 5-HT levels were quantified by comparison to a standard curve (six concentrations ranging between 25 and 400 nM) after normalization for injection volume and extraction efficiency, using the internal standard dihydroxybenzylamine (200 nM). Protein content was measured in an aliquot of the extract, using a colorimetric assay based on the Lowry method. The 5-HT levels were expressed per amount of protein in the sample (mg/ml). Analysis was performed by Statserver 2000. The ED_{50} is the dose at which the compound has 50% of its maximal inhibitory effect on pCA-induced 5-HT depletion in the prefrontal cortex. The absolute 5-HT concentrations are expressed as % effect. 0% effect is defined as the 5-HT levels after pCA + vehicle treatment, 100% effect is defined as the 5-HT levels after vehicle + vehicle. Both control levels were determined in each experiment. When different doses were tested in more than 1 experiment, % effect of test compound was determined based on the control levels in the same experiment. A sigmoidal dose–response curve was fitted to the % effect levels at the log-transformed tested doses and the ED_{50} was derived from the fitted curve.

5.2.5. Ex vivo occupancy. *Drug treatment and tissue preparation*. Male Wistar rats (200 g) were treated by subcutaneous (sc) or oral (po) administration of vehicle or R226161 at four dosages ranging from 0.04 to 10 mg/kg body weight (sc dosages: 0.04, 0.16, 0.63, 2.5 mg/kg; po dosages: 0.16, 0.63, 2.5, 10 mg/kg; three animals per dose). The animals were decapitated 1 h after drug administration. Brains were immediately removed from the skull and rapidly frozen in dry-ice cooled 2-methylbutane (−40 °C). Twenty micrometer-thick sections were cut using a Leica CM 3050 cryostat microtome (van Hopplynus, Belgium) and thaw-mounted on microscope slides (SuperFrost Plus Slides, LaboNord, France). The sections were then kept at −20 °C until use. After determination of ED_{50}-values, a time-course occupancy of the 5-HT transporters and α_2-adrenoceptors by R226161 was performed. The compound was given subcutaneously (0.16 and 2.5 mg/kg) and orally (0.63 and 10 mg/kg) to male Wistar rats (200 g). The animals were sacrificed at specific time points (1, 2, 4, 8 and 16 h, three rats per time point) after administration and brains were processed as described above.

Ex vivo radioligand binding. Occupancy of 5-HT transporters by R226161 was measured in the frontal cortex of each individual rat. After thawing, sections were dried under a cold stream of air and then incubated at room temperature (rt) for 10 min with 1 nM [^{3}H]citalopram in Tris–HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl. Non-specific binding was measured in adjacent sections in presence of 10 μM fluoxetine. To stop the incubation, the slides were washed (2 × 10 min) in Tris–HCl buffer (50 mM, pH 7.4) at 4 °C followed by a rapid dip in cold distilled water. Occupancy of α_2-adrenoceptors by R226161 was measured in the entorhinal cortex of each individual rat. After thawing, sections were dried under a cold stream of air and then incubated at rt for 10 min with 1 nM [^{3}H]idazoxan in Tris–HCl buffer (50 mM, pH 7.4). Non-specific binding was measured in adjacent sections in presence of 10 μM phentolamine. To stop the incubation, the slides were washed (2 × 1 min) in Tris–HCl buffer, pH 7.4, at 4 °C followed by a rapid dip in cold distilled water.

Quantitative autoradiography and data analysis. After application of copper strips to the back surface of the slides, the slides were placed in the β-imager (Biospace, Paris) for 1 h. Acquired images were quantified using the Beta vision program (Biospace, Paris). Specific binding was given as the difference between total binding and non-specific binding measured in adjacent sections. Since only unoccupied receptor remains available for the radioligand, ex vivo receptor labelling is inversely proportional to the receptor occupancy by the in vivo administered drug. Percentages of receptor occupancy by the drug administered to the animal correspond to 100% minus the percentage of receptor labelling in the treated animal. For the determination of ED_{50}-values, the percentage of receptor occupancy was plotted against dosage and the sigmoidal log dose–effect curve of best fit was calculated by non-linear regression analysis, using the GraphPad Prism program. From these

dose–response curves, the ED_{50} (the drug dose producing 50% receptor occupancy) were calculated, with their 95% confidence limits.[25]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.03.053.

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