

N-Oxide analogs of WAY-100635: new high affinity 5-HT_{1A} receptor antagonists

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Abstract—WAY-100635 [*N*-(2-(1-(4-(2-methoxyphenyl)piperazinyl)ethyl))-*N*-(2-pyridinyl)cyclohexanecarboxamide] **1** and its *O*-desmethyl derivative DWAY **2** are well-known high affinity 5-HT_{1A} receptor antagonists, which when labeled with carbon-11 (β^+ ; $t_{1/2} = 20.4$ min) in the carbonyl group are effective radioligands for imaging brain 5-HT_{1A} receptors with positron emission tomography (PET). In a search for new 5-HT_{1A} antagonists with different pharmacokinetic and metabolic properties, the pyridinyl *N*-oxide moiety was incorporated into analogs of **1** and **2**. NOWAY **3**, in which the pyridinyl ring of **1** was oxidized to the pyridinyl *N*-oxide, was prepared via nucleophilic substitution of 2-[4-(2-methoxyphenyl)piperazin-1-yl]ethylamine on 2-chloropyridine-*N*-oxide followed by acylation with cyclohexanecarbonyl chloride. 6Cl-NOWAY **4**, a more lipophilic (pyridinyl-6)-chloro derivative of **3**, was prepared by treating 1-(2-methoxyphenyl)-4-(2-(2-(6-bromo)aminopyridinyl-*N*-oxide)ethyl)piperazine with cyclohexanecarbonyl chloride for acylation and concomitant chloro for bromo substitution. NEWWAY **5**, in which the 2-hydroxy-phenyl group of **2** is replaced with a 2-pyridinyl *N*-oxide group with the intention of mimicking the topology of **2**, was prepared in five steps from 2-(chloroacetylaminopyridine). *N*-Oxides **3–5** were found to be high affinity antagonists at 5-HT_{1A} receptors, with **3** having the highest affinity and a K_i value (0.22 nM) comparable to that of **1** (0.17 nM). By calculation the lipophilicity of **3** (Log $P = 1.87$) is lower than that of **1** by 1.25 Log P units while TLC and reverse phase HPLC indicate that **3** has slightly lower lipophilicity than **1**. On the basis of these encouraging findings, the *N*-oxide **3** was selected for labeling with carbon-11 in its carbonyl group and for evaluation as a radioligand with PET. After intravenous injection of [*carbonyl*-¹¹C]**3** into cynomolgus monkey there was very low uptake of radioactivity into brain and no PET image of brain 5-HT_{1A} receptors was obtained. Either **3** inadequately penetrates the blood–brain barrier or it is excluded from brain by an active efflux mechanism. Rapid deacylation of **3** was not apparent in vivo; in cynomolgus monkey plasma radioactive metabolites of [*carbonyl*-¹¹C]**3** appeared less rapidly than from the radioligands [*carbonyl*-¹¹C]**1** and [*carbonyl*-¹¹C]**2**, which are known to be primarily metabolized by deacylation. Ligand **3** may have value as a new pharmacological tool, but not as a radioligand for brain imaging.

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1. Introduction

WAY-100635 **1** [*N*-(2-(1-(4-(2-methoxyphenyl)piperazinyl)ethyl))-*N*-(2-pyridinyl)cyclohexanecarboxamide] and

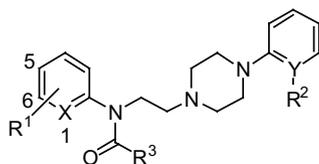
its *O*-desmethyl analog **2** are well-known high affinity 5-HT_{1A} receptor antagonists^{1–6} (Chart 1). [*carbonyl*-¹¹C]**1** is the most popular radioligand for extensive in vivo studies of 5-HT_{1A} receptors in human brain with positron emission tomography (PET),^{5,6} while [*carbonyl*-¹¹C]**2** is a radioligand with increasing popularity because of its enhanced brain entry.^{7,8} These radioligands are giving new insight into the involvement of these receptors in neuropsychiatric disorders (e.g., depression, anxiety, and schizophrenia)^{5,6} and are also useful for drug development.⁹ In human subjects

Keywords: WAY-100635; 5-HT_{1A} Receptor antagonist; High affinity; Pyridinyl *N*-oxide; PET; Aromatic nucleophilic substitution.

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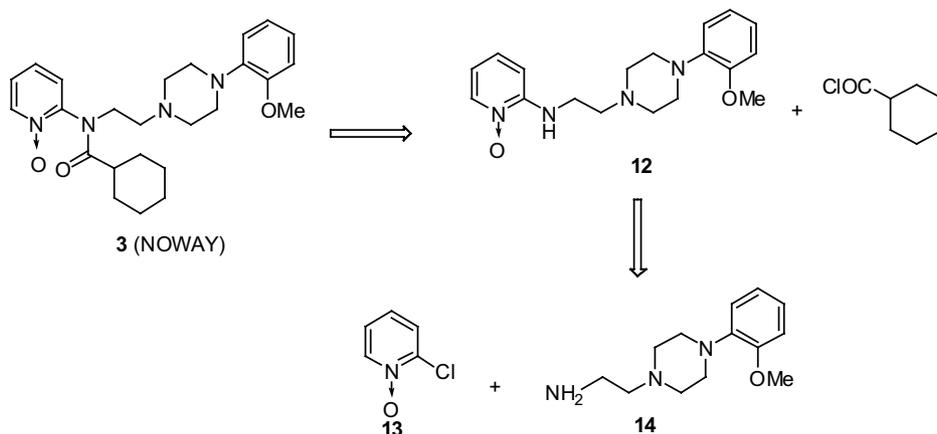
[carbonyl- ^{11}C]**1** and [carbonyl- ^{11}C]**2** are rather rapidly metabolized by amide hydrolysis and are rapidly cleared from plasma.^{8–10} These features detract from the successful use of mathematical models based on the use of an arterial input function for obtaining regional binding potentials for the radioligand in human brain.^{11–13} Therefore, we sought to develop new analogs of **1** and **2** that might retain their pharmacological profile, but which might have more favorable pharmacokinetics and metabolism.

Oxidation of a pyridinyl nitrogen to the *N*-oxide can have various effects on drug-like behavior,¹⁴ often giving drugs with good oral bioavailability,^{15,16} pharmacokinetic properties,^{15,16} solubility,¹⁵ and metabolic stability.^{15,17} Affinity at the target receptor or binding site may also be increased^{18,19} or reduced.²⁰ Formation of the *N*-oxide is a common route of phase I metabolism for drugs containing pyridinyl moieties, and often these metabolites may contribute to drug action.^{13,14} Thus, *N*-oxide analogs of **1** and **2** might be interesting ligands for the 5-HT_{1A} receptor. We aimed to prepare such analogs,



Ligand	X	Y	R ¹	R ²	R ³	
WAY-100635	1	N	C	-	OMe	c.hexyl
DWAY	2	N	C	-	OH	c.hexyl
NOWAY	3	N=O	C	-	OMe	c.hexyl
6Cl-NOWAY	4	N=O	C	6-Cl	OMe	c.hexyl
NEWWAY	5	N	N	-	O	c.hexyl
<i>trans</i> -FCWAY	6	N	C	-	OMe	<i>trans</i> -4-F-c.hexyl
<i>p</i> -MPPF	7	N	C	-	OMe	4-F-phenyl
6FPWAY	8	N	C	6-F	OMe	c.hexyl
6BPWAY	9	N	C	6-Br	OMe	c.hexyl
6IPWAY	10	N	C	6-I	OMe	c.hexyl
5BPWAY	11	N	C	5-Br	OMe	c.hexyl

Chart 1. Structures of 5-HT_{1A} receptor ligands.



Scheme 1. Retrosynthetic analysis of **3** (NOWAY).

to assess their behavior as ligands at the 5-HT_{1A} receptor and, if merited, to evaluate their potential as PET radioligands. The target compounds (**3–5**) were simply the pyridine-*N*-oxide of **1** (compound **3**), the (*pyridinyl*-6)-chloro derivative of **3** (compound **4**) and compound **5** in which the 2-hydroxyphenyl group of **2** is replaced with a 2-pyridinyl *N*-oxide group (compound **5**) (Chart 1).

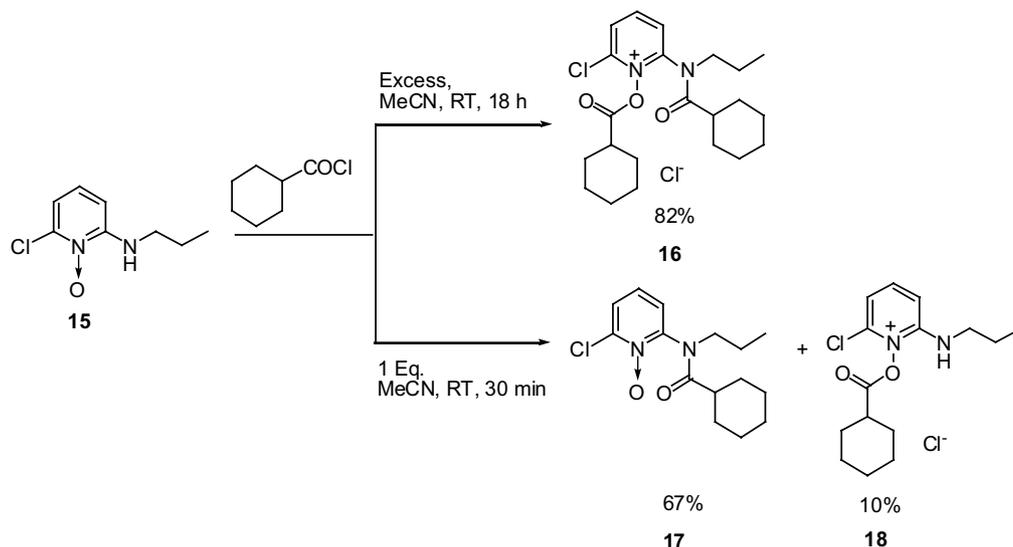
2. Results and discussion

2.1. Chemistry

Retrosynthetic analysis of the *N*-oxide **3** indicated that the pyridine-*N*-oxide **12** might be a useful precursor (Scheme 1). The syntheses of similar pyridine-*N*-oxide derivatives via nucleophilic substitutions in 2-halo-pyridine-*N*-oxides with the amine **14** and subsequent acylation with cyclohexanecarbonyl chloride have been described.²¹

During acylation a side reaction might be expected because of the nucleophilicity of the oxygen in the *N*-oxide moiety. For example, Deady and Stanborough²² found that the *N*-oxide moieties in pyridin-2-amine-*N*-oxides are more reactive than corresponding amines toward acylation. In their study, *O*-acylation was followed by a rapid intramolecular rearrangement, involving a favored five-center transition state to provide the *N*-acyl compound.

Acylation was studied on a model compound, 6-chloro-*N*-propylpyridin-2-amine-*N*-oxide **15**, to find acceptable reaction conditions (Scheme 2). Acylation was first performed with an excess of cyclohexanecarbonyl chloride (50 molequiv) in order to detect and identify an *O*-acyl moiety. The *N,O*-diacyl compound **16** was isolated in 82% yield. One molar equivalent of acid chloride gave a mixture of the *N*-acyl **17** and *O*-acyl **18** compounds, the major product being **17**. Reaction time slightly influenced the ratio of the two products (i.e., 67% of **17** and 10% of **18** after 30 min, and 50% of **17** and 25% of **18** after 22 h).

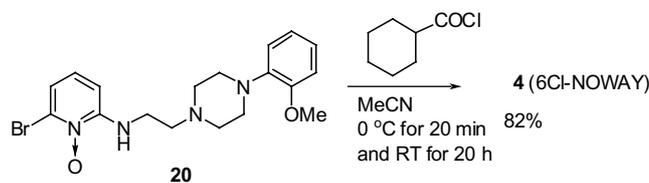


Scheme 2. Acylation of the *N*-oxide **15**.

NOWAY **3** was prepared via the aromatic nucleophilic substitution²¹ of 2-(4-(2-methoxyphenyl)piperazin-1-yl)ethylamine **14** on 2-chloropyridine-*N*-oxide **13** to give compound **12** in 30% yield, followed by acylation with cyclohexanecarbonyl chloride for 55 min to give about a 3:1 mixture of **3** (*N*-acyl) and **19** (*O*-acyl) products in moderate yield (**3** plus **19**, 53%) (Scheme 3); not all starting material **12** was consumed.

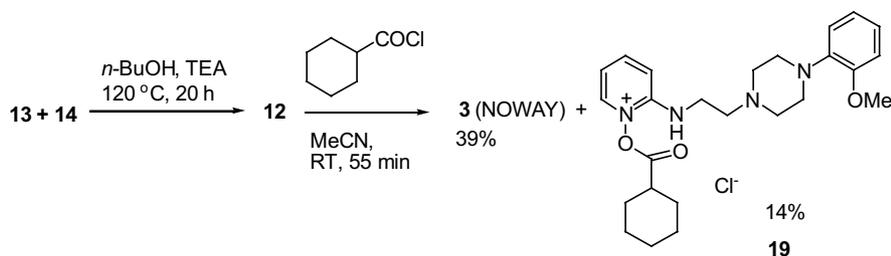
The two *O*-acyl compounds **18** (Scheme 2) and **19** (Scheme 3) could be isolated by TLC and studied with IR but **19** was so unstable that no ¹H NMR spectrum could be recorded. The *N*-acyl compounds **17** (Scheme 2) and **3** (Scheme 3) were also isolated by TLC and were stable when kept dry under an inert atmosphere.

The synthesis of 6Cl-NOWAY **4** was achieved similarly by acylation of the 2-bromopyridine-*N*-oxide amine **20**²³ with cyclohexanecarbonyl chloride at 0°C for 20 min and then at rt for 20 h. Chlorinated ligand **4** was obtained in 82% yield (Scheme 4). Different reaction conditions were tried but none gave the expected bromo analog. The bromine atom at the 6-position of the pyridine-*N*-oxide was immediately exchanged by chloride ion even in the presence of an excess of triethylamine (TEA). Low temperature (0°C) suppressed any formation of the *O*-acyl analog.

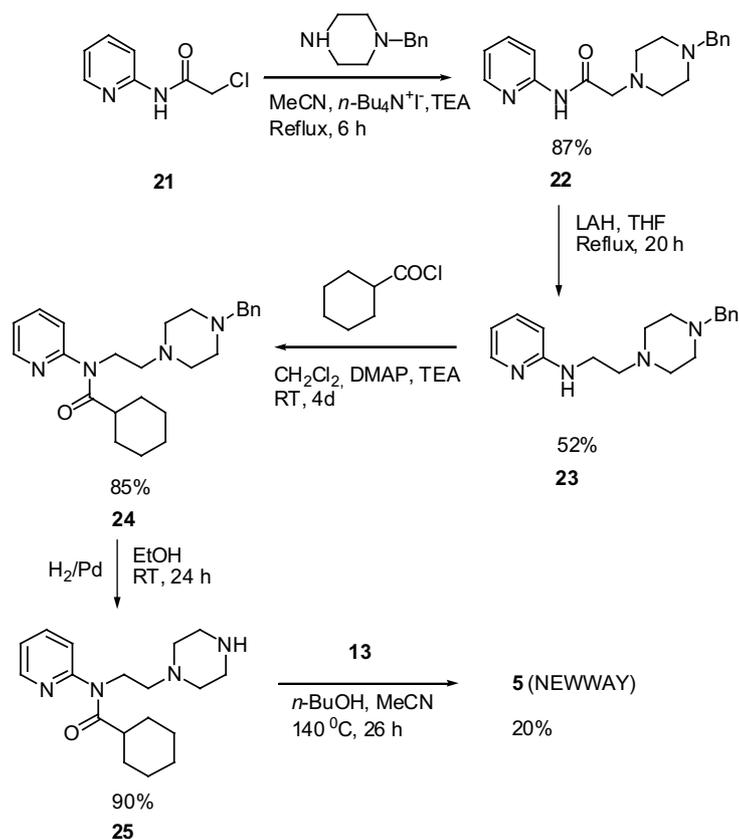


Scheme 4. Synthesis of 6Cl-NOWAY (**4**).

The pyridinyl *N*-oxide group is considered isosteric with certain substituted phenyl groups (e.g., nitro-phenyl), and therefore replacement of a substituted phenyl group with a pyridinyl-*N*-oxide group has been considered a strategy for exploring structure–activity relationships in drug development.^{24,25} NEWWAY **5** was designed to replace the (2-hydroxy)phenyl moiety of **2** by a 2-pyridinyl *N*-oxide moiety. The strategy for the synthesis of **5** was to install the pyridine-*N*-oxide in the last step to prevent any nucleophilic attack of the *N*-oxide group during synthesis. A benzyl group was chosen for protection of the piperazinyl moiety. The synthesis (Scheme 5) started from 2-chloro-*N*-pyridin-2-yl-acetamide **21**.²⁶ Nucleophilic substitution with *N*-benzylpiperazine, reduction with lithium aluminum hydride (LAH), and acylation with cyclohexanecarbonyl chloride gave the benzyl intermediate **24** in 38% overall



Scheme 3. Acylation of the *N*-oxide **12**; synthesis of NOWAY (**3**).



Scheme 5. Synthesis of NEWWAY (5).

yield (three steps). Ligand **5** was obtained after removal of the protecting benzyl group to give **25**, and aromatic nucleophilic substitution of the chloro group in the 2-chloropyridine-*N*-oxide **13** with the piperazinyl group of **25**.

2.2. Pharmacology

Compounds **3–5** were assessed for their ability to inhibit the binding of [³H]5-carboxamido-tryptamine ([³H]5-CT) to human cloned 5-HT_{1A} (*h*5-HT_{1A}) receptors in vitro (Table 1). NOWAY **3** displayed high affinity for *h*5-HT_{1A} receptors, similar to the high affinity of **1** and **2**, while 6Cl-NOWAY **4** displayed 10-fold less affinity and NEWWAY **5** almost 200-fold less affinity. The high affinity of **3** shows the spatial tolerance of the 5-HT_{1A}

receptor for substitutions on the pyridinyl ring of **1** (Table 2). This tolerance has previously been noted for halogen substitutions for hydrogen in the 5- and 6-positions (Table 2; compounds **8–11**).^{27,28} Tolerance depends on the nature of the halogen in the 6-position and, in the case of bromine, distance from the pyridinyl nitrogen. These variations may be due to steric and/or electronic effects.

The ability of each new ligand **3–5** to inhibit the production of cAMP in the presence of forskolin was also assessed on *h*5-HT_{1A} receptors with serotonin (5-HT) (1 μM). Ligands **3–5** displayed complete antagonism in vitro (no intrinsic efficacy).

2.3. Lipophilicity

Lipophilicity is a key predictor of plasma protein binding, blood–brain barrier (BBB) penetration, and the level of brain nonspecific binding of a PET radioligand in vivo.²⁹ The lipophilicities of ligands **1–5** were estimated by computation of Log *P* and Log *D* from their structures (Table 1). The values for the *N*-oxides decrease in the order **4**, **3**, **5** and are all lower than the corresponding values for **1** and **2**. The calculated Log *P* values for **3** and **5** are, respectively, close to and within the desirable range (2.5–3.5) for a compromise between good BBB penetration and low nonspecific binding in brain.²⁹ Chromatographic mobility, especially in reverse phase HPLC, may provide a good experimental index of lipophilicity. In TLC (silica gel; CH₂Cl₂–MeOH; 9:1 v/v)

Table 1. In vitro binding affinities and calculated Log *P* and Log *D* values for ligands **1–5**

Ligand	Affinity <i>K</i> _i (nM)	Log <i>P</i> (Pallas)	Log <i>D</i> ^a (Pallas)
WAY-100635 1	0.17	3.28	2.54
DWAY 2	n.d. ^b	2.77	2.03
NOWAY 3	0.22	1.87	1.14
6Cl-NOWAY 4	1.6 (<i>n</i> = 6)	2.69	1.95
NEWWAY 5	33 (<i>n</i> = 5)	1.25	0.85

^a Log of overall equilibrium distribution between octanol and phosphate buffer at pH 7.4, uncorrected for ionization.

^b Not determined. IC₅₀ = 2.1 ± 0.52 nM and pA₂ = 8.0;³ IC₅₀ = 1.4 nM.⁴ Cf. Ligand **1**: IC₅₀ = 0.37 ± 0.02 nM.³

Table 2. Effect of substituents at the pyridine ring of **1** on affinity at $h5\text{-HT}_{1A}$ receptors and on maximal brain uptake in cynomolgus monkey brain

Ligand	Pyridinyl ring substituent(s)	K_i (nM)	Maximal brain uptake ^a (% ID)	Reference
WAY-100635 1	—	0.029	5.0 at 2 min	This work, 21
NOWAY 3	1-O	0.028	0.65 at 1 min	This work
6Cl-NOWAY 4	6-Cl, 1-O	0.93	n.m.	This work
6FPWAY 8	6-F	10.0	8.0 at 10 min	21,27
6BPWAY 9	6-Br	4.30	5.2 at 5 min	21,27
6IPWAY 10	6-I	10.00		28
5BPWAY 11	5-Br	2.80	n.m.	21,27

n.m. = not measured. Data are uncorrected for radioactivity in blood.

^a Measured with intravenously administered [^{11}C]labeled ligand with PET.

analysis, ligands **1**, **3**, and **4** had R_f values of 0.72, 0.5, and 0.5, respectively, so indicating **3** and **4** to be somewhat less lipophilic than **1**, in accord with the calculated $\text{Log } P/\text{Log } D$ values. When aqueous ammonia was added to the mobile phase or when TLC was performed on an aluminum oxide layer, ligands **1** and **3** showed the same mobility, indicating the same lipophilicity. Ligand **3** showed only slightly lower shorter retention time (6.3 min) than ligand **1** under several reverse phase conditions (e.g., on $\mu\text{-Bondapak C-18}$ column eluted with $\text{MeCN-0.01 M H}_3\text{PO}_4$, 35:65 v/v). Thus, by consideration of calculated $\text{Log } P/\text{Log } D$ values and chromatographic mobility, ligands **3** and **4** appear overall to have only somewhat lower lipophilicity than **1**. Compound **5** was calculated to have substantially lower $\text{Log } P$ and $\text{Log } D$ values than **1** or **2** and was not assessed chromatographically.

The *prima facie* properties of ligand **3** [high affinity, antagonist action (i.e., no efficacy), moderate lipophilicity, low molecular weight, and a site for radiolabeling] are very similar to those of **1** and **2**, and they suggested that **3**, when labeled with carbon-11 (β^+ ; $t_{1/2} = 20.4$ min) might be an effective radioligand for PET imaging of

brain 5-HT_{1A} receptors. Hence, we set out to evaluate [^{11}C]**3** as a potential PET radioligand in cynomolgus monkey. Ligand **3** was successfully labeled in its carbonyl position with carbon-11 in 80% decay-corrected radiochemical yield according to a previously described ^{11}C -acylation technique,^{30,31} but using amine **12** as precursor.

2.4. PET experiments

[^{11}C]**3** was used in two separate PET experiments in a single cynomolgus monkey, one in which the radioligand was given alone (baseline experiment) and one in which the radioligand was given after treatment of the monkey with **1** at a dose known to block monkey brain 5-HT_{1A} receptors^{7,32} (pre-treatment experiment).

No image of brain 5-HT_{1A} receptors was obtained after intravenous injection of [^{11}C]**3** in either PET experiment due to a very low whole brain uptake of radioactivity (Fig. 1). This was about 0.54% of injected dose at about 2.5 min, steadily declining to about 0.32% of injected dose at 90 min in the baseline experiment,

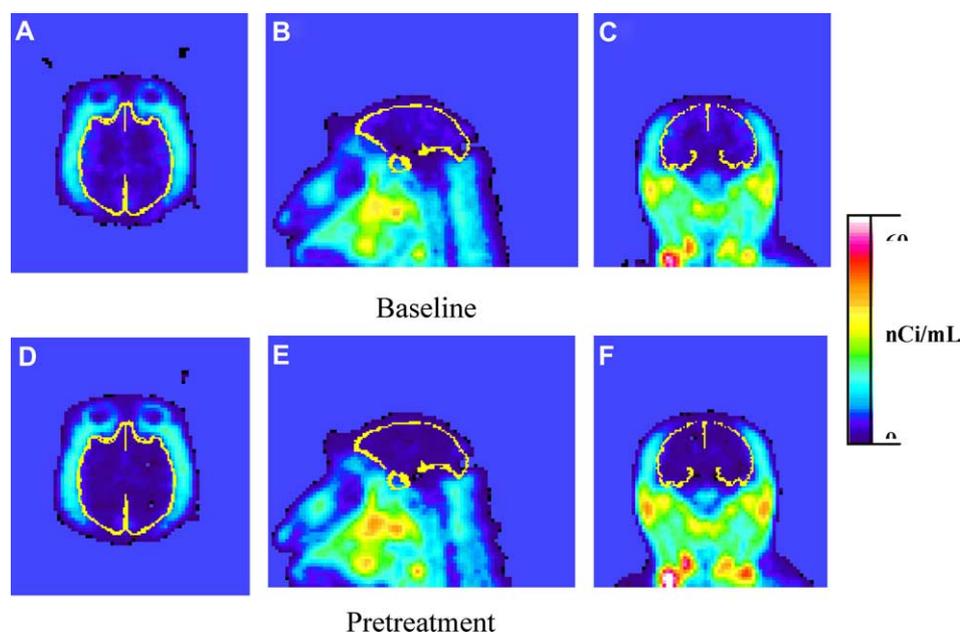


Figure 1. Horizontal (panels A and D), sagittal (panels B and E), and coronal (panels C and F) PET images of cynomolgus monkey head after intravenous injection of NCA [^{11}C]NOWAY in the baseline experiment (panels A–C) and pre-treatment experiment (D–F). Images are from emission data summed from 9 min after injection.

and about 0.59% of injected dose at about 2.5 min, steadily declining to about 0.15% of injected dose at 90 min in the pre-treatment experiment (Fig. 2). Time-radioactivity curves for cerebellum were very similar in each experiment. The higher whole brain radioactivity (as % of injected dose) between 25 and 90 min in the baseline experiment compared to that in the pre-treatment experiment may indicate some generalized but very low level of 5-HT_{1A} receptor-specific binding. It should be noted that these data are uncorrected for blood radioactivity content. For comparison [*carbonyl*-¹¹C]**1** and some halo analogs maximally enter cynomolgus monkey brain to a strikingly greater extent, namely about 5–8% of injected doses at 2.5–8 min after injection^{7,10} (Table 2). The somewhat lower lipophilicity of **3**, compared to **1**, may not account alone for the poor ability of **3** to enter monkey brain.

Ligand **1** and the structurally related ligand, *p*-MPPF (*N*-(2-(1-(4-(2-methoxyphenyl)piperazinyl)ethyl))-*N*-(2-pyridinyl)*p*-fluorophenylcarboxamide, **7**) are known to be substrates for rat brain P-glycoprotein (Pgp),³³ a pump which can exclude certain compounds (substrates) from brain. Indeed, **7** has significantly lower brain uptake than **1** because it is so much better a substrate for elimination from brain by Pgp.³⁴ Thus, it may reasonably be expected that **3** is also a substrate for brain Pgp, based on its very close structural similarity to **1** and **7**.

Only a few studies have examined the uptake of *N*-oxides into rodent or primate brain directly. The brain uptakes of clozapine and its *N*-oxide metabolites have been examined in mice.³⁵ At 10 min after *i.p.* administration, clozapine has 24-fold higher brain uptake than the *N*-oxide. A further study has shown that clozapine *N*-oxide is unable to cross the BBB in rat,³⁶ though *N*-oxidation is the main route of clozapine metabolism in rat brain.³⁷ The *N*-oxide of Lu 25-109 (5-ethyl-2*H*-tetrazol-5-yl-1,2,3,6-tetrahydro-methylpyridine), a M1 muscarinic agonist, does not cross the BBB in rat, unlike Lu 25-

109 itself.³⁸ For compounds related to Lu 25-109, Log *D* did not predict BBB penetration, indicating the possible role of transporter proteins in uptake and/or elimination from brain. These reports and our findings suggest that Pgp and/or other transporter proteins (pumps) may generally exclude *N*-oxides from brain, perhaps as part of a 'drug efflux-metabolism alliance'.³⁹ The possibility that **3**, by virtue of its structural similarity to **1** and **7** and its *N*-oxide moiety, is a very good substrate for Pgp or other transporter was not however investigated further in this study.

In the baseline and pre-treatment PET experiments, the proportions of radioactivity in monkey plasma represented by parent radioligand were, respectively, 80% and 94% at 5 min, 55% and 55% at 30 min, and 46% and 46% at 45 min. [*carbonyl*-¹¹C]**1** and [*carbonyl*-¹¹C]**2** are each primarily metabolized by deacylation (amide hydrolysis),^{8,10,†} and this is the expected route of metabolism for [*carbonyl*-¹¹C]**3**. The HPLC analysis of the radioactive metabolites of [*carbonyl*-¹¹C]**3** is consistent with this expectation, since only more polar radioactive metabolites were observed. Comparison of the limited data from the two experiments with [*carbonyl*-¹¹C]**3** and the averaged data published for [*carbonyl*-¹¹C]**1** from six monkeys,¹⁰ suggests that [*carbonyl*-¹¹C]**3** is less rapidly metabolized than [*carbonyl*-¹¹C]**1** in cynomolgus monkey.

3. Conclusions

Three new high affinity antagonists of the *h*5-HT_{1A} receptor **3–5** were discovered. Among these, the *N*-oxide **3** is notable in having lipophilicity and high affinity that are similar to those of the well known antagonists, **1** and **2**. Ligand **3** however does not enter brain to any great extent in monkey and when labeled with carbon-11 gives no PET image of the distribution of brain 5-HT_{1A} receptors. Ligand **3** is possibly a good substrate for Pgp or another transporter protein that actively excludes it from brain. [*carbonyl*-¹¹C]**3** appears to have a slower rate of appearance of radioactive metabolites in plasma than [*carbonyl*-¹¹C]**1** and [*carbonyl*-¹¹C]**2**. Ligand **3** may have potential value as a new pharmacological tool but not as a radioligand for brain imaging. In ligand **4** the 6-chloro group increased lipophilicity but reduced affinity for the 5-HT_{1A} receptor by almost 7-fold; this shows the tolerance of the 5-HT_{1A} receptor for substituents in both the 1- and 6-positions of the pyridinyl ring of **1**. Ligand **5** has almost 200-fold lower affinity for the 5-HT_{1A} receptor than **2**. Hence, the *N*-2-pyridinyl moiety is not an especially good bioisosteric mimic for the

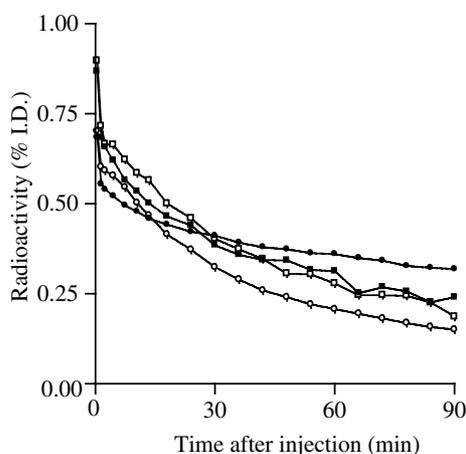


Figure 2. Time courses for radioactivity (% injected dose) in monkey whole brain and cerebellum after injection of NCA [*carbonyl*-¹¹C]NOWAY: ● cerebellum in baseline experiment; ○ cerebellum in pre-treatment experiment; ■ whole brain in baseline experiment; □ whole brain in pre-treatment experiment.

[†]From this study, the possibility that [*carbonyl*-¹¹C]**1** is metabolized in vivo via rapid oxidation to its *N*-oxide, followed by intramolecular *trans*-*O*-acylation and *O*-deacylation might be considered. However, after the injection of [*carbonyl*-¹¹C]**1** into cynomolgus monkey, no radioactive *N*-oxide has been specifically detected in plasma with radio-HPLC.¹⁰ Also careful investigations of other radioligands that are closely related in structure to **1** (e.g., *trans*-FCWAY; **6**) have not revealed any pyridinyl-*N*-oxides forming as metabolites in human or liver microsomes.^{40,41}

2-hydroxyphenyl group in DWAY **2** or the *N*-(2-methoxyphenyl) groups in WAY **1**, which are recognized as being important structural features for 5-HT_{1A} receptor binding.⁴²

4. Experimental

4.1. General

4.1.1. Materials. Chemical names follow IUPAC nomenclature. WAY-100635 **1** was prepared as described previously.⁴³ 2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethylamine **14**,⁴⁴ 2-chloropyridine-*N*-oxide **13**,⁴⁵ 1-(2-methoxyphenyl)-4-(2-(2-(6-bromo)aminopyridinyl-*N*-oxide)ethyl)piperazine **20**,²¹ and 2-chloro-*N*-pyridin-2-yl-acetamide **21**²⁶ were prepared as reported. All other chemicals were obtained commercially (Aldrich, Acros or Fluka) and used without purification.

4.1.2. Analytical, spectroscopic, and chromatographic methods. Melting points were determined on a digital melting point apparatus (Electrothermal) and are uncorrected.

Elemental analyses were performed by the Microanalytical Laboratory of the University of Groningen and were within $\pm 0.4\%$ of the theoretical values.

IR spectra were obtained on an ATI-Mattson spectrometer.

¹H NMR spectra were obtained in CDCl₃ on a Varian Gemini (300 or 200 MHz) instrument at 300 K. ¹³C NMR spectra were obtained in CDCl₃ on a Varian Gemini (50 MHz) instrument at 300 K. Chemical shifts are reported in δ values (ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard (CDCl₃, $\delta = 7.24$ ppm in ¹H NMR and $\delta = 77$ ppm in ¹³C NMR). Signals are described as s, d, t, dd, m, br for singlet, doublet, triplet, double doublet, multiplet, and broad, respectively.

Mass spectra (ES) were recorded on a Sciex API 3000 instrument or on a Unicam 610/Automass 150 GC-MS system (EI). HRMS data were obtained by the Microanalytical Department of the University of Groningen on a Jeol MS Route JMS600H instrument.

Analytical thin layer chromatography (TLC) was performed on silica gel layers (60 PF 254 pre-coated aluminum sheets, 0.2 mm layer; Merck) or aluminum oxide layers (60 F 254 neutral pre-coated aluminum sheets, 0.2 mm layer; Merck). Preparative TLC was performed on silica gel (60 PF 254 pre-coated glass plate; 1 or 2 mm layer; Merck).

4.2. Chemistry

4.2.1. 1-(2-Methoxyphenyl)-4-(2-(2-aminopyridinyl-*N*-oxide)ethyl)piperazine (12**).** 2-Chloropyridine-*N*-oxide **13** (2.41 g; 18.6 mmol) and TEA (2.59 mL; 18.6 mmol) were added to 2-[4-(2-methoxyphenyl)piperazin-1-

yl]ethylamine **14** (4.38 g; 18.6 mmol) in *n*-BuOH (80 mL). The mixture was heated at 120 °C for 20 h and then the reaction stopped by evaporation of solvent. Column chromatography (silica gel; CH₂Cl₂-MeOH, 95:5 v/v) provided pure **12** (1.83 g; 30%). The hydrochloride salt was then prepared by adding HCl (1 M in Et₂O; 4 equiv) and recrystallized from absolute EtOH. IR (neat): 1625, 1569, 1498, 1241 (N→O) cm⁻¹; ¹H NMR (300 MHz, CDCl₃): 8.05 (d, *J* = 6 Hz, 1H), 7.13 (t, *J* = 8 Hz, 1H), 7.05 (br s, 1H), 6.78–6.94 (m, 4H), 6.49–6.57 (m, 2H), 3.80 (s, 3H), 3.31–3.37 (m, 2H), 3.00–3.07 (m, 4H), 2.68–2.74 (m, 6H); ¹³C NMR (50 MHz, CDCl₃): 139.60, 136.00, 127.00, 121.50, 119.50, 116.80, 109.90, 109.55, 104.35, 54.95, 53.85, 51.80, 48.95, 37.50; MS (EI): 328 [M]⁺; Anal. Calcd for C₁₈H₂₄N₄O₂: C, 65.83; H, 7.37; N, 17.06. Found: C, 65.39; H, 7.35; N, 16.70.

4.2.2. General acylation procedure (on **12, **15**, and **20**).** Cyclohexanecarbonyl chloride (1 molequiv) was added to a solution of amine (1 molequiv) in MeCN under N₂. The mixture was stirred at rt or at 0 °C (ice-water bath) and then the reaction quenched with aq Na₂CO₃ solution (10% w/v). Product was extracted with CH₂Cl₂, dried over MgSO₄, filtered, and concentrated.

4.2.2.1. *N*-(2-(1-(4-(2-Methoxyphenyl)piperazinyl)-ethyl))-*N*-(2-pyridinyl-*N*-oxide)cyclohexanecarboxamide, NOWAY (3**).** Acylation was performed on **12** (33 mg; 99 μ mol) at rt. Preparative TLC (0.2 mm layer; CH₂Cl₂-MeOH, 9:1 v/v) gave pure **3** (17 mg, 39%). IR (neat): 1673 (amide), 1496, 1451, 1240 (N→O) cm⁻¹; ¹H NMR (200 MHz, CDCl₃): 8.26–8.30 (m, 1H), 7.51–7.56 (m, 1H), 7.24–7.31 (m, 2H), 6.81–7.03 (m, 4H), 4.13 (m, 1H), 3.83 (s, 3H), 2.99 (br s, 4H), 2.65 (br s, 6H), 1.03–1.94 (m, 12H); ¹³C NMR (50 MHz, CDCl₃): 175.20, 150.65, 146.40, 139.40, 138.70, 125.70, 124.20, 123.40, 121.55, 119.40, 116.55, 109.70, 54.45, 53.85, 51.60, 48.70, 41.55, 41.25, 24.05; MS (ES): 439 [M+H]⁺.

4.2.2.2. 1-(2-Methoxyphenyl)-4-(2-(2-(1-cyclohexanecyloxyppyridinium)amino)ethyl)piperazine chloride (19**).** Acylation was performed on **12** (33 mg; 99 μ mol) at 0 °C. Preparative TLC (0.2 mm layer; CH₂Cl₂-MeOH, 9:1 v/v) gave pure **19** (6 mg; 14% yield). IR (neat): 1730 (NO-COC₆H₁₁), 1458 cm⁻¹; MS (ES): 439 [M+H]⁺.

4.2.2.3. *N*-(6-Chloro-1-cyclohexanecyloxyppyridinium-2-yl)-*N*-propylcyclohexanecarboxamide chloride (16**).** Acylation was performed on **15** (20 mg; 0.1 mmol). Preparative TLC (0.2 mm layer; CH₂Cl₂-MeOH, 95:5 v/v) gave pure **16** (39 mg, 82%). IR (neat): 1705 (NO-COC₆H₁₁) cm⁻¹; ¹H NMR (300 MHz, CDCl₃): δ 7.46–7.49 (dd, *J* = 3 Hz, *J* = 7 Hz, 1H), 7.15–7.21 (m, 2H), 2.22–2.32 (m, 4H), 1.10–1.90 (m, 22H), 0.82 (t, *J* = 7 Hz, 3H).

4.2.2.4. *N*-(6-Chlorooxidopyridin-2-yl)-*N*-propylcyclohexanecarboxamide (17**).** Acylation was performed on **15** (20 mg; 0.1 mmol) at rt. Preparative TLC (0.2 mm layer; CH₂Cl₂-MeOH, 95:5 v/v) gave pure **17** (21.5 mg; 67%). IR (neat): 1670 (amide), 1272 (N→O)

cm^{-1} ; ^1H NMR (300 MHz, CDCl_3): δ 7.46 (dd, $J = 3$ Hz, $J = 7$ Hz, 1H), 7.11–7.21 (m, 2H), 1.36–1.85 (m, 11H), 0.901.19 (m, 4H), 0.82 (t, $J = 8$ Hz, 3H); MS (EI): 296–298 $[\text{M}]^+$.

4.2.2.5. (6-Chloro-1-cyclohexaneacyloxypyridinium-2-yl)-*N*-propyl chloride (18). Acylation was performed on **15** (20 mg; 0.1 mmol) at rt. Preparative TLC (0.2 mm layer; CH_2Cl_2 –MeOH, 95:5 v/v) gave pure **18** (3.3 mg; 10%). IR (neat): 3284 (amine), 1707 (NOCOC₆H₁₁), 1621, 1559 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3): δ 7.06–7.21 (m, 2H), 6.68 (d, $J = 8$ Hz, 1H), 6.46 (d, $J = 8$ Hz, 1H), 3.16–3.22 (m, 2H), 2.21–2.28 (m, 1H), 1.86 (d, $J = 13$ Hz, 2H), 1.59–1.70 (m, 5H), 1.17–1.44 (m, 5H), 0.96 (t, $J = 7$ Hz, 3H).

4.2.2.6. *N*-(2-(1-(4-(2-Methoxyphenyl)piperazinyl)-ethyl))-*N*-(2-(6-chloropyridinyl)-*N*-oxide)cyclohexanecarboxamide, 6Cl-NOWAY (4). Acylation was performed on **20** (65 mg; 0.16 mmol). Preparative TLC (1 mm layer; CH_2Cl_2 –MeOH, 90:10 v/v) gave pure **4** (62 mg; 82%). IR (neat): 1678 (amide), 1597, 1500, 1240 (N→O) cm^{-1} ; ^1H NMR (300 MHz, CDCl_3): δ 7.43 (m, 2H), 7.12 (m, 1H), 6.95–6.76 (m, 4H), 4.00 (s, 1H), 3.76 (s, 3H), 2.92 (br s, 4H), 2.62 (br s, 6H), 1.83–0.96 (m, 12H); ^{13}C NMR (50 MHz, CDCl_3): δ 175.30, 150.65, 147.90, 141.20, 139.30, 124.45, 123.35, 122.80, 121.65, 119.45, 116.60, 109.70, 54.50, 53.85, 51.55, 48.85, 48.50, 41.70, 41.25, 28.55, 27.60, 24.30, 24.05; MS (ES): 473 $[\text{M}+\text{H}]^+$.

4.2.3. 2-(4-Benzyl-piperazin-1-yl)-*N*-pyridin-2-yl-acetamide (22). *N*-Benzylpiperazine (5.10 g; 29 mmol), TEA (12.1 mL; 8.78 g; 87 mmol; 3 equiv) and *n*-Bu₄NI (535 mg; 1.45 mmol; 0.05 equiv) were added to 2-chloro-*N*-(pyridin-2'-yl)-acetamide **21** (5.42 g; 31.9 mmol; 1.10 equiv) in MeCN (250 mL). The mixture was refluxed for 6 h and the reaction stopped by evaporation of the solvent. The residue was dissolved in CH_2Cl_2 . The organic layer was successively washed with H₂O, aq Na₂CO₃ (10% w/v), and brine. The organic layer was dried over Na₂SO₄, filtered, and concentrated. Purification by column chromatography (silica gel; CH_2Cl_2 –MeOH, 98:2 v/v) provided pure **22** (8.61 g; 87%). IR (neat): 3309, 1698, 1574, 1508, 1432 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3): 9.54 (br s, 1H), 8.27 (d, $J = 4$ Hz, 1H), 8.19 (d, $J = 8$ Hz, 1H), 7.66 (dt, $J = 2$ Hz, $J = 9$ Hz, 1H), 7.28–7.19 (m, 5H), 6.99 (m, 1H), 3.49 (s, 2H), 3.11 (s, 2H), 2.60 (s, 4H), 2.52 (s, 4H); ^{13}C NMR (50 MHz, CDCl_3): 168.00, 146.50, 136.80, 127.65, 126.80, 125.65, 118.35, 112.40, 61.40, 60.65, 52.10, 51.50; MS (EI): 310 $[\text{M}]^+$. HRMS (EI): Calcd for C₁₈H₂₂N₄O: 310.1793, obsd: 310.1790; Anal. Calcd for C₁₈H₂₂N₄O: C, 69.65; H, 7.14; N, 18.05. Found: C, 69.41; H, 7.15; N, 17.92.

4.2.4. *N*-[2-(4-Benzyl-piperazin-1-yl)-ethyl]-pyridin-2-yl-amine (23). LAH (50 mg; 1.36 mmol; 2 equiv) was added to 2-(4-benzyl-piperazin-1-yl)-*N*-pyridin-2-yl-acetamide **22** (210 mg; 0.68 mmol) in THF (25 mL). The mixture was refluxed for 20 h under N₂. Reaction was stopped by addition of H₂O and NaOH (4 M). The mixture was then refluxed for 10 min and extracted with ethyl acetate.

The organic layers were dried over Na₂SO₄, filtered, and concentrated. Preparative TLC (1 mm layer; CH_2Cl_2 –MeOH, 9:1 v/v) gave pure **23** (105 mg; 52%). IR (neat): 3368, 1602, 1494, 1444 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3): 8.01 (d, $J = 4$ Hz, 1H), 7.34 (t, $J = 7$ Hz, 1H), 7.26–7.17 (m, 5H), 6.49 (t, $J = 6$ Hz, 1H), 6.32 (d, $J = 9$ Hz, 1H), 5.10 (br s, 1H), 3.45 (s, 2H), 3.28 (m, 2H), 2.56 (t, $J = 5$ Hz, 2H), 2.44 (m, 8H); ^{13}C NMR (75 MHz, CDCl_3): 156.25, 145.60, 135.50, 134.80, 126.70, 125.70, 124.55, 110.15, 104.55, 60.50, 54.15, 50.45, 50.35, 36.00; MS (EI): 202, 189, 146, 119.

4.2.5. *N*-(2-(1-(4-(Benzyl)piperazinyl)ethyl))-*N*-(2-pyridinyl)cyclohexanecarboxamide (24). Cyclohexanecarbonyl chloride (29 μL ; 43.2 mg; 0.29 mmol; 1.2 equiv), TEA (51 μL ; 37 mg; 0.37 mmol; 1.5 equiv), and a catalytic amount of 4-(dimethylamino)pyridine were added successively to [2-(4-benzyl-piperazin-1-yl)-ethyl]-pyridin-2-yl-amine **23** (73 mg; 0.25 mmol) in CH_2Cl_2 (5 mL) at 0°C. The mixture was stirred under N₂ at 0°C for 15 min and then at rt for 4 days. Reaction was stopped by addition of H₂O and Na₂CO₃. The mixture was extracted with dichloromethane and the organic layers dried over Na₂SO₄, filtered, and concentrated. Preparative TLC (three plates, 0.2 mm layers; CH_2Cl_2 –MeOH; 95:5 v/v) gave pure **24** (85 mg; 85%). IR (neat): 1655, 1585, 1466 cm^{-1} ; ^1H NMR (200 MHz, CDCl_3): 8.48–8.52 (m, 1H), 7.78–7.69 (m, 1H), 7.30–7.18 (m, 7H), 3.93 (t, $J = 7$ Hz, 2H), 3.47 (s, 2H), 2.57–2.16 (m, 11H), 1.71–1.43 (m, 7H), 1.25–1.02 (m, 3H); ^{13}C NMR (50 MHz, CDCl_3): 174.75, 147.70, 136.55, 136.45, 127.70, 126.65, 125.50, 120.75, 120.65, 61.50, 54.55, 51.50, 43.70, 40.85, 28.00, 24.10; MS (ES): 407 $[\text{M}+\text{H}]^+$; HRMS (EI): Calcd for C₂₇H₃₆N₄O: 406.2732, obsd: 406.2718.

4.2.6. *N*-(2-(1-Piperazinyl)ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide (25). Palladium on charcoal (10 mg; 10%) was added to *N*-(2-(1-(4-(benzyl)piperazinyl)ethyl))-*N*-(2-pyridinyl)cyclohexanecarboxamide **24** (40 mg; 98 μmol) in absolute EtOH (6 mL). A hydrogen balloon was attached to the flask and the air purged by successive evacuation and hydrogen fills. The mixture was stirred at rt for 24 h. Reaction was stopped by filtration over Celite, which was then rinsed with ethanol. Preparative TLC (silica gel; CH_2Cl_2 –MeOH; 90:10 v/v) gave pure **25** (28 mg; 90% yield). IR (neat): 2928, 1655, 1584, 1466, 1437 cm^{-1} ; ^1H NMR (200 MHz, CDCl_3): 8.52 (dd, $J = 2$ Hz, $J = 6$ Hz, 1H), 7.76 (dt, $J = 2$ Hz, $J = 8$ Hz, 1H), 7.22–7.27 (m, 2H), 4.75 (br s, 1H), 4.30 (t, $J = 6$ Hz, 2H), 2.84 (m, 4H), 2.49 (m, 6H), 2.21 (m, 1H), 1.93–0.84 (m, 10H); ^{13}C NMR (50 MHz, CDCl_3): 174.90, 154.35, 147.80, 136.65, 120.75, 120.65, 95.45, 54.95, 51.80, 43.85, 43.40, 40.85, 28.00, 24.05; MS (ES): 317 $[\text{M}+\text{H}]^+$.

4.2.7. *N*-(2-(1-(4-(2-Pyridinyl)-*N*-oxide)piperazinyl)-ethyl))-*N*-(2-pyridinyl)cyclohexanecarboxamide NEW-WAY (5). 2-Chloropyridine-*N*-oxide **13** (21 mg; 0.16 mmol) and TEA (33 μL ; 24 mg; 0.24 mmol; 1.5 equiv) were added to *N*-(2-(1-piperazinyl)ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide **25** (50 mg; 0.16 mmol) in *n*-BuOH (3 mL) plus CH₃CN (3 mL).

The mixture was heated at 140 °C for 26 h. Reaction was then stopped by evaporation of the solvent. Preparative TLC (four plates, 0.2 mm layers; CH₂Cl₂–MeOH, 90:10 v/v) gave pure **5** (12.6 mg; 20%). IR (neat): 1653 (amide), 1584, 1500, 1437, 1242 (N→O) cm⁻¹; ¹H NMR (300 MHz, CDCl₃): 8.47 (d, *J* = 4 Hz, 1H), 8.08 (d, *J* = 6 Hz, 1H), 7.70 (dt, *J* = 2 Hz, *J* = 7 Hz, 1H), 7.21–7.11 (m, 3H), 6.80–6.74 (m, 2H), 3.93 (t, *J* = 7 Hz, 2H), 3.28 (br s, 4H), 2.62–2.53 (m, 6H), 2.17 (m, 1H), 1.88 (br s, 1H), 1.70–0.79 (m, 9H); ¹³C NMR (50 MHz, CDCl₃): 147.85, 139.15, 136.65, 125.70, 120.65, 116.20, 113.35, 54.60, 51.10, 46.10, 43.50, 40.90, 28.05, 24.10; MS (ES): 410 [M+H]⁺.

4.3. In vitro receptor binding assays

Membranes from HeLa cells expressing *h5*-HT_{1A} receptors are incubated in tris buffer (50 mM; pH 7.7) with [³H]5-CT (4.5 nM) for 15 min at rt in the presence of the test compound. Nonspecific binding was determined by adding methiothepine (10 μM). Incubation was terminated by rapid filtration through unifilter GF/B filters using a Tomtec cell harvester. Filters were counted in a Wallac Scintillation counter and data were processed using Prism (GraphPad).

4.4. Efficacy assays

HeLa cells expressing *h5*-HT_{1A} receptor (*B*_{max} = 50 fmol/mg protein) were washed and then incubated for 20 min with the test compound **3–5** at concentrations up to 10 μM and a fixed concentration of 5-HT (1 μM). Incubation was terminated by aspiration and cAMP was extracted with ice-cold HCl. The amount of cAMP in each sample was measured in a Flashplate (RIA-based system). Data were processed using Prism (GraphPad).

4.5. Lipophilicity

The Log *P* and Log *D* values of **1–5** were calculated from the Pallas 3.0 program (CompuDrug International Inc., San Francisco, CA, USA). Lipophilicity was also assessed with TLC (silica gel; CH₂Cl₂–MeOH, 9:1 v/v) for **1**, **3**, and **4** and also with reverse phase HPLC analysis on a μ-Bondapak C-18 column (300 × 3.9 mm o.d.; particle size 10 μm) eluted at mL/min with MeCN–0.01 M H₃PO₄ (65:35 v/v) at 3 mL/min for **1** and **3**.

4.6. Preparation of [*carbonyl*-¹¹C]**3**

NCA [*carbonyl*-¹¹C]cyclohexanecarbonyl chloride was produced from cyclotron-produced [¹¹C]carbon dioxide, exactly as described previously.^{46,47} The preparation of [*carbonyl*-¹¹C]**3** followed a procedure analogous to that used formerly to produce [*carbonyl*-¹¹C]**1** and other analogs.^{30,31} Thus, the *N*-oxide **12** (9.6 mg; 29 μmol) was treated with NCA [*carbonyl*-¹¹C]cyclohexanecarbonyl chloride in tetrahydrofuran (500 μL) containing TEA (40 μL) for 4 min and then separated by reverse phase HPLC on a μ-Bondapak column (300 × 3.9 mm o.d.; 10 μm particle size; waters) eluted with MeOH–0.1 M

ammonium formate–TEA (570:430:3 by vol at 6 mL/min and formulated by evaporation of mobile phase and dissolution in saline for injection).

4.7. Analysis of [*carbonyl*-¹¹C]**3**

The radiochemical purity of [*carbonyl*-¹¹C]**2** was determined on a sample of the final formulation with HPLC on a μ-Bondapak-C18 column (300 × 3.9 mm o.d.; particle size, 10 μm; waters) eluted with MeCN–0.01 M phosphoric acid (30:70 v/v) at 3 mL/min with eluate monitored for radioactivity (Beckman β-flow detector) and absorbance at 270 nm. [*carbonyl*-¹¹C]**3** (retention time, 6.3 min) was identified by co-injection with authentic **3**.

4.8. PET experiments with NCA [*carbonyl*-¹¹C]**3** in cynomolgus monkey

The study was approved by the Animal Ethics Committee of Northern Stockholm. A male cynomolgus monkey (6.53 kg) was anaesthetized by repeated intramuscular injection of ketamine–xylazine [Ketarlar[®]; 1–2 mg/kg per h]–(Rompun[®]; 1 mg/kg per h) and then positioned in a Siemens ECAT EXACT HR PET camera (resolution: 3.8 mm full width half maximum) so that the transaxial imaging planes of the head were parallel to the cantomeatal line. The monkey head was fixed in position during the PET scans.⁴⁷ Monkey body temperature was maintained with a thermostatically controlled heating pad. NCA [*carbonyl*-¹¹C]**3** (51 MBq) was injected intravenously into the left sural vein and regional cerebral radioactivity uptake was measured in 3-D mode for up to 90 min and corrected for physical decay. Data were obtained from 47 sections with a separation of 3.3 mm. The whole brain contour was defined in situ according to an atlas of cryosected cynomolgus monkey head.⁴⁶ Radioactivity was calculated from the sequence of time frames, corrected for physical decay and plotted versus time. The radioactivity concentration in the ROI for the whole brain was multiplied by the brain volume (estimated to be ~65 mL) to calculate the percent of injected radioactivity in whole brain. The calculated value for radioactivity in the brain, as percent of injected dose, was then divided by the radioactivity injected and multiplied by 100. In a second experiment, NCA [*carbonyl*-¹¹C]**3** (51 MBq) was injected intravenously at 10 min after injection of **1** (0.5 mg/kg; i.v.) into the same monkey.

4.9. Measurement of unchanged [*carbonyl*-¹¹C]**3** and radioactive metabolites in monkey plasma

The percentage of radioactivity in plasma as unchanged radioligand was determined by an HPLC method that has been shown to be effective for several other PET radioligands.^{47,48} After intravenous injection of [*carbonyl*-¹¹C]**3** into monkey, arterial blood samples were taken at 5, 30, and 45 min during the PET scan. Each blood sample was centrifuged at 2000g for 1 min. The supernatant plasma (0.5 mL) was deproteinized with acetonitrile (0.7 mL) that had been pre-spiked with reference **3**.

The radioactivity of this mixture was measured in a well counter and a portion (1 mL) analyzed by injection onto a gradient HPLC system, a μ -Bondapak-C18 column (300 \times 7.8 mm o.d.; 10 μ m particle size) eluted at 6.0 mL/min with acetonitrile–0.01 M phosphoric acid.^{47,48} Over 98% of the radioactivity in the blood sample was recovered in the deproteinized plasma taken for analysis.

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