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N-CHLOROMETHYL QUINUCLIDINIUM DERIVATIVES: A NEW CLASS OF IRREVERSIBLE LIGANDS FOR 5-HT$_3$ RECEPTORS.

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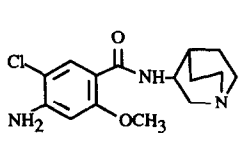
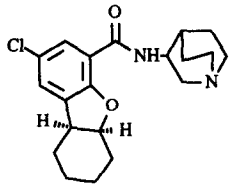
Abstract: *The quinuclidine ring of potent 5-HT$_3$ receptor antagonists such as zacopride and the 1,8-naphthalimide derivatives 3 reacts with methylene chloride at room temperature to produce the corresponding chloromethyl quaternary derivatives. The compounds derived from (S)-zacopride and (R)-3 are potent ligands for 5-HT$_3$ receptor as evaluated in binding assays. Incubated with entorhinal cortex membranes, they produced a dose-dependent decrease in the B_{max} value for [^{3}H]-BRL-43694 which was inhibited by zacopride or GR 38032F. The quaternary derivative of (R)-3 is a promising tool for studying 5-HT$_3$ receptors due to its high affinity and fluorescent properties.*

Recently, several structural analyses of the 5-HT$_3$ receptor antagonists have provided a three dimensional model of the 5-HT$_3$ receptor pharmacophore[1-5]. However, little information is available about the chemical groups in the peptidic sequence of the binding site for agonists or antagonists of the 5-HT$_3$ receptor. This is due to the unique fact that the 5-HT$_3$ receptor is a ligand-gated-ion channel[6] similar to NMDA or nicotinic receptors for which the neurotransmitter binding sites are supposed to be located on the extracellular part of the α subunit[7]. This situation is in contrast to that of the other serotonin receptor subtypes, for which a 3D-model has been proposed, based on the homology of their peptidic sequences with that of the rhodopsin receptor[8]. Several experiments have confirmed the role of particular amino acids in the transmembrane helices in the binding of agonists or antagonists[9] to various 5-HT receptor and on the basis of this model Hibert proposed a complete structural description of several serotonin receptor subtypes. For the 5-HT$_3$ receptor, such a structural analysis is impossible since only the α subunit has been cloned and the peptidic sequence described[10], but no information is available on the tertiary structure of the extracellular part of this peptidic chain. A first step towards this goal would be the synthesis of specific, irreversible ligands capable of binding covalently with the receptor site. We describe here the results of our investigation in this field and report the preparation of the first irreversible 5-HT$_3$ receptor antagonists.

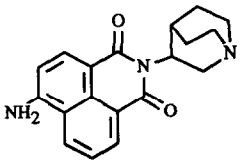
Among 5-HT$_3$ receptor antagonists, a number of them possess the quinuclidine ring indicating a particularly good fit between the receptor and this heterocycle. The first compounds synthesized were members of the benzamide family such as zacopride **1**[11] or RG 12915 **2**[12] and they display enantioselectivity for the 5-HT$_3$ receptor since the (S) enantiomers are the most potent compounds[13].



Zacopride 1



RG 12915 2



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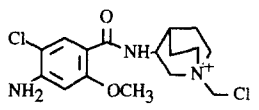
Recently, we reported[14] a new family of potent 5-HT$_3$ receptor antagonists derived from the naphthalimide moiety where the most potent compounds possess the quinuclidine ring as in compound **3**. In contrast to the benzamide derivatives, we observed a reversal of the receptor

enantioselectivity, the (R) enantiomer being the more potent isomer. We postulated the existence of a second hydrogen donor group[15] for the binding of the additional carbonyl group in the receptor site to explain this improved recognition of the (R) isomer. During the synthesis of zacopride **1** or the naphthalimide derivatives **3**, we observed the formation of an insoluble compound when these derivatives were heated with methylene chloride during the purification or crystallisation steps. It has already been reported that this solvent can react with the basic nitrogen atom of a heterocyclic system[16] to give a water-soluble quaternary derivative and should not be used for the crystallisation and purification of such derivatives. More recently, a cyclisation reaction in a family of antidopaminergic benzamide derivatives was reported with methylene chloride [17]. A reappraisal of this reaction seemed worthwhile with our compounds. We observed that the (R) and (S) enantiomers of **1** and **3**, in methylene chloride at room temperature, provided precipitates in good yield which were identified as the quaternary derivatives **4** and **5**.



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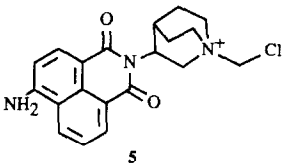

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Their structures were identified by ^1H NMR and ^{13}C NMR spectra, mass spectrometry and quantitative determination of chlorine. In particular, the presence of the $-CH_2Cl-$ group linked to the basic nitrogen was clearly demonstrated by the chemical shifts of the CH_2 group ((R)- or (S)-**4**, ^1H NMR (CD_3OD), δ_{CH2}: 5.29; ^{13}C NMR (CD_3OD) δ_{CH2}: 69.3 ; (R)- or (S)-**5**, ^1H-NMR (CD_3OD), δ_{CH2}: 5.26; ^{13}C-NMR (CD_3OD) δ_{CH2}: 69.8) and by the high solubility of the compounds in water. The affinity for these compounds for 5-HT_3 receptors was evaluated by binding assays with [^3H]-BRL 43694 using rat entorhinal cortex[18] and the results are shown in Table I.

5-HT_3 RECEPTOR BINDING AFFINITY DETERMINED IN RAT ENTORHINAL CORTEX

Reference compounds	K_i±SEM, nM[a]	Compounds	α^{25}(°)[c]	K_i±SEM, nM[a]
(R)-zacopride	2.6±0.4	(R)-4	-39.5	20.1±3.7
(S)-zacopride	0.2±0.04	(S)-4	+45.4	14.8±3.8
(R)-3	0.15±0.04	(R)-5	-86	0.35±0.04
(S)-3	23.5±4	(S)-5	+94	328±50

Table I. a) [^3H]-BRL-43694 was used in the binding assays which were carried out using rat entorhinal cortex (30 min-25°C) and seven concentrations of the competing compounds. Each assay was done in triplicate and inhibition curves were analyzed by a computer-assisted fitting program (ALLFIT). K_i values were determined from the Cheng-Prussof equation. c) The optical rotation was measured using an Hg Ray (436 nm) in MeOH (C=1).

The data indicate the unfavorable influence of a quaternary substituent on the nitrogen atom of the zacopride derivatives **4** since a drop in the range of one order of magnitude was observed for both enantiomers. This result is similar to that reported for zacopride methyl iodide[19]. On the other hand, the (R) naphthalimide derivative **5** was equipotent to the parent compound, whereas a marked decrease in the affinity was seen for the (S)-**5** isomer. Thus, we again observed with these new naphthalimide derivatives a potent affinity for the 5-HT_3 receptor, an inversion in the recognition of the chirality with regard to the benzamides and an increase in the enantioselectivity of 5-HT_3 receptors since the (R) enantiomer is 3 orders of magnitude more potent than the (S) compound. It seemed to us that the (S)-**4** and (R)-**5** derivatives could be useful pharmacological tools for elucidating the structure of the 5-HT_3 receptor binding site. In a preliminary experiment, entorhinal cortex membranes were incubated with a fixed dose of (S)-**4** or (R)-**5** and a decrease in the B_{max} value was observed when saturation curves were performed with [^3H]-BRL 43694, suggesting the formation of an irreversible bond between the ligand and the receptor site. Several experiments were carried out to support these preliminary results and the data are reported in Table II.

RESULTS OF SCATCHARD PLOTS FOR [^{3}H]-BRL-43694 AFTER INCUBATION OF ENTORHINAL CORTEX MEMBRANES WITH DIFFERENT DOSES OF (S)-4 and (R)-5[a].

Concentration of compound	B_{max} (fmol/mg prot)	K_d (nM) [^{3}H]-BRL 43694
Control	29.5	1.24
(S)-4 (10^{-5}M)	16.9	1.89
(S)-4 (10^{-4}M)	0	n.d[b]
Control	23.8	1.85
(R)-5 (10^{-8}M)	11.2	0.84
(R)-5 (10^{-5}M)	5.1	0.46

Table II. a) Membrane preparations, stored at -80°C were thawed, homogenized and suspended in phosphate buffer (0.2M, pH 6.6). They were incubated at 25°C for 30 min with various concentrations of the drugs and centrifuged at 40000 g for 10 min at 0-5°C. The pellets were washed three times by resuspension in 10 vol of buffer and centrifugation. The final pellets were suspended in 10 vol of 50 mM Hepes (pH:8.4) and the saturation curve was calculated using nine concentrations of [^{3}H]-BRL-43694 (0.15-4.2nM). The samples (0.5ml) were incubated at 25°C for 30 min. Non-specific binding was determined from samples incubated with 10 µM of GR 38032F. Triplicate determinations was made for each point. Data, Scatchard representations and K_D and B_{max} values were analyzed and calculated by non-linear and linear computer-assisted curve fitting software (GRAPH PAD and ALLFIT). b) not determined.

A dose-dependent decrease in the B_{max} value was observed with both compounds, but the naphthalimide derivative, (R)-**5** was more potent than the zacopride derivative, (S)-**4**. A reduction of 50% in the initial B_{max} value was obtained using 10^{-8}M (R)-**5**, whereas a similar decrease was only seen with a 1000 fold higher concentration of (S)-**4**. It was postulated that the observed decrease in the B_{max} value was due to the formation of an irreversible bond between the molecule and the binding site. To support this hypothesis, it was shown, in additional experiments, that 3 or 6 washes of the membranes after the incubation period were without influence on the decrease in the B_{max} value. In addition, total protection against alkylation of the binding site was produced by the addition of GR 38032F or zacopride (10^{-5}M) during the incubation period. The mechanism of the alkylation could be due to a reaction of the nucleophilic part of the receptor binding site with the N^+-CH_2-Cl moiety followed by fragmentation of the quinuclidine ring giving rise to an electrophilic species capable of reacting with the site. Several experiments are in progress to support this hypothesis.

The interest in (R)-**5** as an important tool to characterize the 5-HT_3 receptor was also indicated by the fluorescent properties of these naphthalimide derivatives. It has been reported[20] that substituted amino-1,8-naphthalimide possesses strong fluorescent properties associated with a good quantum yield. We observed for (R)-**3** and (R)-**5**, compared to the unsubstituted derivatives, a marked coloration of their crystals which became orange due to the presence of an absorption band in the region of 450 nm which was broad and structureless (H_2O, λ_{max}: 430 nm, ϵ/dm^3 mol^{-1}cm^{-1}= 22500) and a marked fluorescence (λ_{max}: 550 nM). The relevance of developing a fluorescent probe for studies of receptor localisation has been reported recently and the potential to visualize membrane-bound receptors at a higher resolution than that possible with classical autoradiography techniques has been shown with such ligand[21]. In summary, the (R)-**5** compound, with its high affinity for 5-HT_3 receptor, its ability to bind covalently to the receptor binding site and its fluorescent properties, constitutes a promising tool to examine the localisation and distribution of 5-HT_3 receptors and experiments are in progress on this compound and the series of related derivatives.

Acknowledgments

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