Radioligand binding studies: Structure affinity relationships of antagonists at muscarinic, serotonergic and histaminergic receptor subtypes

Radioligandbindungsstudien: Struktur-Wirkungs-Beziehungen von Antagonisten an muskarinischen, serotonergen und histaminergen Rezeptor Subtypen

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1 Introduction

1.1 Muscarinic receptors

At the beginning of the 20th century it was suggested for the first time that the physiological effects elicited by acetylcholine (ACh) (Fig. 1.3) were mediated by two different receptor populations, later named nicotinic and muscarinic receptors according to their stimulating agents nicotine and muscarine (Fig. 1.3), respectively (Dale, 1914). Nicotinic receptors turned out later to be members of the family of ligand-gated ion channels and were soon further divided into a neuronal and muscular subtype family (for review see Paterson and Nordberg, 2000). In the case of muscarinic receptors, diversity was more complex and clarification needed until the end of the 1980's. First reports about heterogeneity within the muscarinic receptors were given in the 1950's with the cardio-selective compound gallamine (Riker and Wescoe, 1951) and later with the ganglionic stimulant McN-A-343 (Roszkowski, 1961). With the development of pirenzepine the first compound displaying real selectivity was found (Hammer et al., 1980) and the existence of at least two distinct muscarinic receptors was clearly confirmed, named M_1 and M_2 (Hammer and Giachetti, 1982). Further reports about additional heterogeneity within the M_2 subtype accumulated, but it was not before cloning techniques came up that the existence of all additional subtypes could be clearly confirmed. Cloning of the porcine M_1 receptor (Kubo et al., 1986) was the beginning of this new era in the field of muscarinic receptors. Ensuing in the following years the cDNA of four additional human receptors, M_2 - M_5, were cloned (Bonner et al., 1987, 1988). The coding regions for $hM_{1\text{-}5}$ receptors were shown to be intronless. The Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) for muscarinic receptors summarised that M_1 - M_4 receptors could be clearly discriminated pharmacologically, with gene products related to a functional property in humans or animals (Caulfield and Birdsall, 1998). In the case of the M_5 subtype, the receptor was cloned, stably expressed in CHO cells and characterised in binding studies (Buckley et al., 1989). However, a functional model for endogenous M_5 receptors is still missing. Actually, there is no evidence for further subtypes within the muscarinic receptor family and it is likely that all muscarinic receptors are identified to date. Recently, the entire genes for the hM_2 and hM_3 receptors were mapped (Zhou et al., 2001; Forsythe et al., 2002).

1.1.1 Signal transduction

Muscarinic receptors were shown to belong to the superfamily of G-protein-coupled receptors (GPCRs) (for review see Wess, 1996) in contrast to nicotinic receptors which belong to the family of ligand-gated ion channels. Fig. 1.1 gives a cartoon of a nicotinic and a muscarinic receptor.

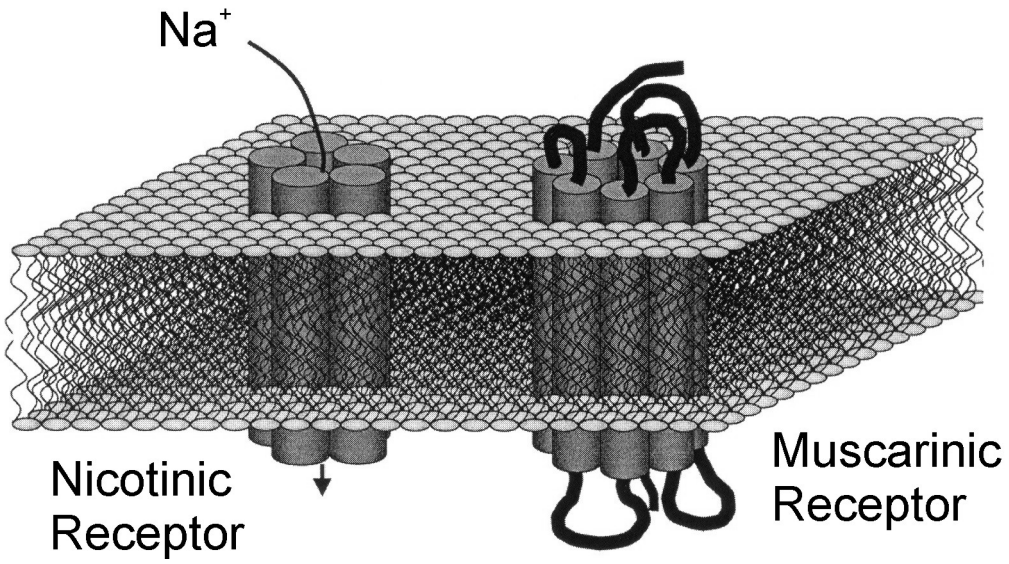


Fig. 1.1 *Model of a nicotinic and muscarinic receptor. Taken from Felder et al., 2000.*

Hydrophobicity analysis and comparison to the identified GPCR structure of rhodopsin (Lu et al., 2002) suggests, that each muscarinic receptor displays seven α-helical membrane-spanning domains (TM1-7), an external N-terminus and an internal C-terminus. $M_{1\text{-}5}$ receptors possess a high degree of sequence homology, especially within the TM domains (Bonner, 1989). Heterogeneity between $M_{1\text{-}5}$ receptor proteins was observed within the large (approximately 240 amino acids long) third intracellular loop (i3), the N- and C-terminus. The most critical receptor domains involved in G-protein-coupling are located at the i2-, i3-loop and C-terminus (Brann et al., 1993; Burstein et al., 1998). For this regions sequence similarity was observed between the odd-numbered and even-numbered subtypes, sharing the same signal transduction pathways (for review see Hulme et al., 1990; Felder et al., 2000). M_1, M_3, and M_5 were shown to couple preferentially via G_q (Fig. 1.2), leading to an activation of phospholipase Cβ (PLCβ) resulting in an enzymatic breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2), thus providing the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to Ca^{2+} liberation

from intracellular stores, inreasing $[Ca^{2+}]_i$ which is a cellular trigger for several downstream effects. DAG activates proteinkinase Cβ (PKCβ) initiating a variety of cellular effects. M_2 and M_4 receptors preferably couple to G_i (Fig. 1.2). Thus, activation leads to inhibition of adenylate cyclase (AC) resulting in decreased intracellular cAMP levels.

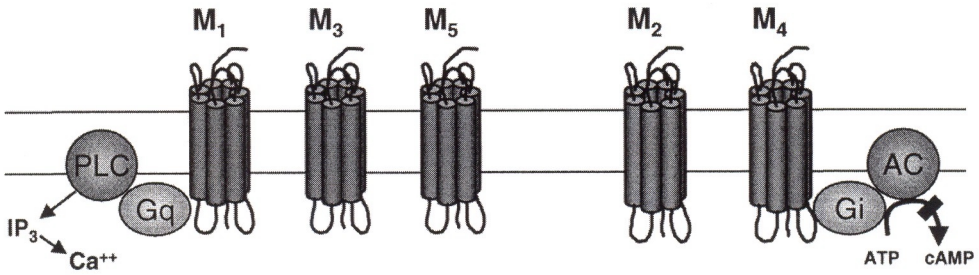


Fig. 1.2 *Preferential second messenger pathways for $M_{1\text{-}5}$ receptors. Cartoon taken from Felder et al., 2000.*

Unfortunately, a vast amount of additional pathways was reported, thus making signal transduction within the muscarinic system a highly complex network (Nathanson, 2000). One should notice, that multiplicity was not only detected within the subunits of G-proteins (17α-, 5β-, and 12γ-subunits were cloned), but also for AC and PLC, with 9 and 11 identified isoenzymes each, respectively (for review see Ulloa-Aguirre et al., 1999; Hur and Kim, 2002). M_2 and M_4 receptors may (when expressed at high levels) couple to PLC (isoenzymes β2 and β3) via their $G_{\beta\gamma}$ subunit, in contrast to the G_q coupled receptors which stimulate PLC (isoenzymes β1-4) via their G_α subunit. The $G_{\beta\gamma}$ subunit was shown to activate K^+ channels in the heart, too. For M_1, M_3, and M_5 receptors stimulation of phospholipase A_2, phospholipase D and tyrosine kinases was shown, but it remains yet to be elucidated whether this is a result of a direct G-protein interaction or a downstream effect of PLC. Regulation of cAMP level via muscarinic receptors is even more complex. G_q-, as well as G_i-coupled subtypes can increase cAMP levels via interaction with G_s (when expressed at high levels). Additionally, cAMP level can be regulated in a cell-type specific manner via second messenger effects on phosphodiesterases.
Oligomerization (for review see Rios et al., 2001; Dean et al., 2001) further complicating the signal transduction was shown for many GPCRs, including muscarinic receptors (Maggio et al., 1999; Park et al., 2001).

1.1.2 Receptor desensitization and sequestration

An interesting feature observed for all muscarinic receptor subtypes is constitutive activity that could be reduced with atropine acting as an inverse agonist. Distinct amino acids within TM3, TM6 and the i2 loop were pinpointed to be involved in the level of constitutive activity (Spalding and Burstein, 2001; Ford et al., 2002). Internalization of muscarinic receptors following agonist stimulation was observed at all subtypes. However, rate and extent of sequestration were depending on the examined subtype and the used cell system (Koenig and Edwardson, 1996). Rapid uncoupling from G-proteins and desensitization was shown in the case of M_2 receptors to be regulated by phosphorylation through G-protein-coupled receptor kinases (GRK) within the i3 loop and arrestins (Hosey et al., 1999). Different mechanisms involved in M_2 receptor sequestration were determined depending on the examined cell type. In JEG-3 cells, but not in HEK293 cells, arrestins were involved in receptor trafficking (Schlador and Nathanson, 1997; Roseberry and Hosey, 1999). The underlying mechanism involved in HEK-293 cells remains yet unclear. In PC12 cells, involvement of the small GTPase Rab11a was demonstrated in M_4 sequestration from the cell surface to endosomes (Volpicelli et al., 2002). Further studies are needed to clarify the mechanisms involved in internalization of muscarinic receptors.

1.1.3 Receptor distribution in the periphery

In the following paragraphs a brief summary of the most important features of muscarinic receptors in peripheral tissues is given.

1.1.3.1 M_1 receptors

This subtype was detected in sympathetic and parasympathetic ganglia where they facilitate ganglionic neurotransmission (Hammer and Giachetti, 1982). Studies with M_1-KO mice showed the increase in heart rate and blood pressure following the application of McN-A-343 (presumably due to catecholamine release from sympathetic ganglia) to be abolished (Hamilton et al., 2001; Hardouin et al., 2002). M_1 receptors were found at neurons within the gastrointestinal tract (GIT). Immunological studies detected this subtype in salivary glands (Levey, 1993), where

they play a minor role in salivation (Bymaster et al., 2003). In rabbit vas deferens M_1 receptors were detected to mediate inhibition of neurogenic contractions (Eltze et al., 1988). Recently, it was shown that M_1 receptors in stomach smooth muscles of M_3-KO mice mediate an NO driven relaxation that is normally masked by a direct M_3-mediated contraction (Stengel and Cohen, 2003).

1.1.3.2 M_2 receptors

M_2 receptors are widely distributed within periphery. In heart tissue, M_2 receptors are the most important subtype, mediating negative chronotropic, inotropic and dromotropic effects (Caulfield, 1993). In studies with M_2-KO mice, these effects were abolished, confirming the predominant role of this subtype in heart tissue (Stengel et al., 2000; Bymaster et al., 2001). Great amounts of M_2 receptors were detected in a variety of smooth muscle preparations (for review see Eglen et al., 1994, 1996) where they are located pre- and postjunctionally. M_2 receptors clearly outnumber M_3 receptors in most smooth muscle tissues. Studies with M_2-KO mice demonstrated a reduced potency of muscarinic agonists to contract smooth muscle preparations (Stengel et al., 2000; Bymaster et al., 2001). However, the role of postjunctional receptors is not fully understood. Prejunctionally located M_2 receptors represent the major subtype among muscarinic autoreceptors (Caulfield, 1993; Langer, 1997).

1.1.3.3 M_3 receptors

This subtype was shown to be located in many smooth muscle preparations including GIT, urinary tract and airways (Fetscher et al., 2002). M_3 receptors were shown to be the most important subtype involved in smooth muscle contraction. In studies wit M_3-KO mice contractile response of smooth muscle was massively impaired in tissue preparations of GIT and urinary bladder (Matsui et al., 2000; Stengel et al., 2002). Additionally, M_3-KO mice (but not $M_{1,2,4,5}$-KO mice) had enlarged pupils, confirming a role for M_3 receptors on the tone of pupillary sphincter muscles (Matsui et al., 2000). M_3 receptors were detected in salivary glands. Studies with M_3-KO mice showed a pronounced decrease in salivation being more important than M_1 and M_4 receptors (Bymaster et al., 2003). Activation of M_3 (and possibly M_1) receptors on pancreatic B-cells resulted in an increased insulin secretion (Verspohl et al., 1990).

1.1.3.4 M_4 receptors

M_4 receptors are located in several peripheral human and animal tissues. However, the physiological role of M_4 receptors often remains unclear. In human detrusor muscle, M_4 receptors serve as inhibitory autoreceptors (D'Agostino et al., 2000). A minor role in salivation could be confirmed in M_4-KO mice (Bymaster et al., 2003). Somadendritically located M_4 receptors mediate NANC relaxation in the rabbit anococcygeus muscle. This preparation is therefore used as a functional M_4 model (Gross et al., 1997). Involvement in autoreceptor function, next to M_2 receptor, was suggested in postganglionic sympathetic axons in mouse bladder (Trendelenburg et al., 2003).

1.1.3.5 M_5 receptors

Apart from its localisation in blood vessels in brain and the periphery, this subtype was not detected in other peripheral tissues (Phillips et al., 1997; Elhusseiny et al., 1999).

1.1.4 Receptor distribution in the CNS

All known subtypes of muscarinic receptors were found to be widely distributed within human brains using different experimental approaches. Radioligand binding studies (Waelbroeck et al., 1989; Ferrari-Dileo et al., 1994), autoradiographic experiments (Rodriguez-Puertas et al., 1997) and immunological studies in rats (Yasuda et al., 1992; Levey, 1993) and humans (Flynn et al., 1995) were carried out. Taken together, highest levels of M_1 receptors were detected in cortex and hippocampus, whereas in the striatum even higher levels of M_4 receptors were identified. In midbrain, pons, cerebellum and brainstem the M_2 subtype was the most frequently expressed receptor. Interestingly, M_3 receptors were detected in low, M_5 receptors in very low levels within the examined brain areas. Because highly selective, brain penetrating ligands for muscarinic receptor subtypes are not commonly available, it is difficult to assign single subtypes to special behavioural and cognitive functions. With the generation of genetically modified mice, lacking one or more muscarinic receptor subtypes (knock-out, KO mice), some central functions could be elucidated and clearly attributed to a single subtype (for review see Bymaster et al., 2003).

1.1.4.1 M_1 receptors

With high density in cortex and hippocampus a role of M_1 receptors in cognition and memory was suggested. Studies with M_1-KO mice showed cognitive dysfunctions in some tasks, confirming a role of this subtype (Anagnostaras et al., 2003). M_1-KO mice displayed hyperactivity with increased locomotor activity and had increased dopaminergic transmissions in the striatum (Gerber et al., 2001). Participation of M_1 receptors located in the hypothalamic suprachiasmatic nucleus (SCN) in the regulation of circadian rhythm was shown in rats (Gillette et al., 2001). In a study using antisense inhibition of the M_1 subtype an involvement in central antinociception was detected (Ghelardini et al., 2000).

1.1.4.2 M_2 receptors

Several aspects concerning the physiological role of this subtype were elucidated. Muscarinic agonists penetrating into the CNS elicit a potent analgesic response. Several studies with M_2-KO, M_4-KO and M_2/M_4-KO mice addressed this aspect and found the M_2 subtype to be most important for the analgesic effects (Gomeza et al., 2001; Duttaroy et al., 2002). Additionally, a minor role of M_4 receptors was determined in analgesic effects. Taken together, M_2 and M_4 receptors mediate muscarinic agonist evoked analgesic effects on spinal and supraspinal levels (for review see Wess et al., 2003). Further studies revealed the M_2 subtype to be critically involved in mediating central muscarinic effects such as tremor and hypothermia following application of a muscarinic agonist (Bymaster et al., 2001; Gomeza et al., 2001). A role within the hypothalamic-pituitary-adrenocortical axis was detected in M_2-KO mice (Hemrick-Luecke et al., 2002). The increase of serum corticosterone levels following the application of a muscarinic agonist was abolished in mice lacking M_2 receptors. One of the perhaps most important physiological M_2 functions in the CNS is participation in release control of ACh as an autoreceptor. The critical role of M_2 was confirmed in several studies using human and rat brains (Feuerstein et al., 1992; Kitaichi et al., 1999; Zhang et al., 2002a). Within cortex and hippocampus, the ACh release is controlled mainly via the M_2 subtype, whereas in the striatum the M_4 subtype plays the predominant role.

1.1.4.3 M_3 receptors

Only little is known about the function of this comparably small receptor fraction. Studies with M_3-KO mice showed a role for M_3 receptors in food intake and control of whole body weight (Yamada et al., 2001b). Mice lacking this subtype were hypophagic and lean with pronounced decreases in leptin and insulin levels. An indirect inhibitory effect on striatal dopamine (DA) release was reported for this subtype (Zhang et al., 2002b).

1.1.4.4 M_4 receptors

Studies revealed a participation in analgesic effects mediated through the muscarinic system next to the M_2 subtype (Duttaroy et al., 2002). A major role in autoinhibitory release control of ACh in the striatum was demonstrated (Zhang et al., 2002a). Further on, the involvement of this subtype in dopaminergic transmission was investigated. M_4-KO mice had increased basal locomotor activity and increased D_1 receptor sensitivity (Gomeza et al., 1999). M_4 receptors facilitate dopamine release in the striatum (Zhang et al., 2002b). An interesting report was given about the influence of steroids on M_4 density in some brain regions. Density of M_4 receptors in hippocampus and hypothalamus were reduced in rats by application of estrogens but not gestagens (El Bakri et al., 2002).

1.1.4.5 M_5 receptors

The years following the discovery of M_5 receptors gave only little insight in the role of this subtype. Reviews summarising the fragmented knowledge were published recently (Reever et al., 1997; Eglen and Nahorski, 2000). It was more than ten years after discovery of this subtype when first M_5-KO studies showed influence on dopaminergic transmission (Yeomans et al., 2001; Forster et al., 2001). M_5 receptors were shown to facilitate a prolonged dopamine release in some brain regions. Mice lacking M_5 receptors showed increased drinking, suggesting a role in fluid intake for this subtype (Takeuchi et al., 2002). Finally, this subtype was shown to be involved in the ACh driven dilation in cerebral, but not peripheral blood vessels via NO (Yamada et al., 2001a).

1.1.5 Non-neuronal ACh

Apart from neuronal cells, ACh and the synthesising enzyme, choline acetyltransferase (ChAT), have been detected in a variety of e.g. epithelial cells, endothelial cells and immune cells (for review see Wessler et al., 2003). It is important to notice, that non-neuronal ACh acts as a local signalling molecule involved in the regulation of cellular functions, whereas neuronal ACh acts as a neurotransmitter, rapidly mediating communication between neurons and effector cells. Table 1.1 gives the most prominent differences between the neuronal and non-neuronal cholinergic systems.

Table 1.1 *Differences between the neuronal and non-neuronal cholinergic systems. Modified from Wessler et al. (2003).*

Neuronal		Non-neuronal
nerve terminal	**SYNTHESIS**	whole cell
vesicles	**STORAGE**	cytosol (?)
exocytosis / on demand	**RELEASE**	transporter / continously
hot spots	**RECEPTORS**	uniformly expressed
short lasting	**ACTION**	continously
rapid	**ELIMINATION**	slow

In contrast to neuronal transmission, ACh in non-neuronal cells is synthesised in the whole cells and not stored in special compartments. A constant release was shown to be mediated by organic cation transporters, whereas a complex exocytosis mechanism takes place during neuronal ACh release.

Comparatively many studies were undertaken with regard to the cholinergic system in lymphocytes (for review see Kawashima and Fujii, 2000). M_4 and M_5 receptors were detected in all studies, whereas expression of M_{1-3} receptors was not detected in all examined persons. There is an increasing body of evidence, that ACh from T-lymphocytes acts as an autocrine and/or paracrine factor regulating immune functions next to the cytokine system. Recently, the involvement of autocrine cholinergic mechanisms on expression of both, IL-2 and its receptor in T-cells was demonstrated (Nomura et al., 2003). Changes in muscarinic receptor density in

lymphocytes were detected in patients suffering from Alzheimer's disease or asthma, giving hope to derive new clinical markers or therapeutic options (Tayebati et al., 2001; Ricci et al., 2002). Increased M_5 mRNA levels were detected in lymphocytes during immunological responses (Fujii et al., 2003). Additionally, all muscarinic receptor subtypes were located in human skin melanocytes (Buchli et al., 2001), but the physiological role remains unclear. Expression of ChAT in human ovary was detected in endocrine cells expressing M_1 and M_5 receptors. A role in cell proliferation was suggested (for review see Mayerhofer et al., 2003). Finally, an important role of the non-neuronal cholinergic system was detected in the eye (for review see Duncan and Collison, 2003), where involvement in tear fluid production (mediated exclusively via M_3) and form-deprived myopia was shown (mediated perhaps via M_1 and M_3).

1.1.6 Muscarinic ligands

A brief overview of muscarinic agonists and antagonists is given in the following paragraphs. Muscarinic ligands have been extensively reviewed elsewhere (Eglen and Watson, 1996; Eglen et al., 1999; Broadley and Kelly, 2001). The development of selective muscarinic ligands turned out to be a difficult task and only in the last years progress was reported.

1.1.6.1 Muscarinic agonists

Several non-selective agonists next to the endogenous ligand ACh are known, including the closely related compounds carbachol and oxotremorine (Fig. 1.3). As hope came up to obtain potent drugs for Alzheimer's disease (AD) therapy with the development of M_1-selective agonists, a rush in pharmaceutical industry started in the search for CNS penetrating, M_1-selective agonists. Several compounds were tested in clinical trials. However, all tested compounds had only little selectivity for M_1 receptors. Additionally, most drugs had a poor bioavailability and were metabolically unstable. Thus, studies with xanomeline, milameline, sabcomeline, talsaclidine and alvameline in AD patients were discontinued due to side effects or little efficacy (for review see Sheardown, 2002). Only one so called "M_1-selective" agonist, cevimeline (Fig. 1.3), was approved to the market but not for use in AD therapy. It was approved

next to the non-specific agonist pilocarpine (Fig. 1.3) for therapy of the autoimmune disease Sjögren's syndrome (for review see Fox et al., 2001; Fox, 2003). This disease leads to dysfunction of salivary and lacrimal glands resulting in dry mouth and eyes (xerostomia). Stimulation of M_1 and M_3 receptors within glandular tissue ameliorates these symptoms. Although most companies stopped their programs for M_1-selective molecules, there is still some research carried out in order to develop second generation compounds with better selectivity, increased bioavailability and metabolic stability (for review see Fisher et al., 2002). One newer M_1-selective compound is AF-150(S) with somewhat improved characteristics (Fig. 1.3). Synthesis of dimeric muscarinic ligands led to the discovery of M_1/M_4 selective compounds (Christopoulos et al., 2001). The idea of using M_1 agonists in AD therapy is not buried yet and improved drug candidates might lead to better results in clinical trials.

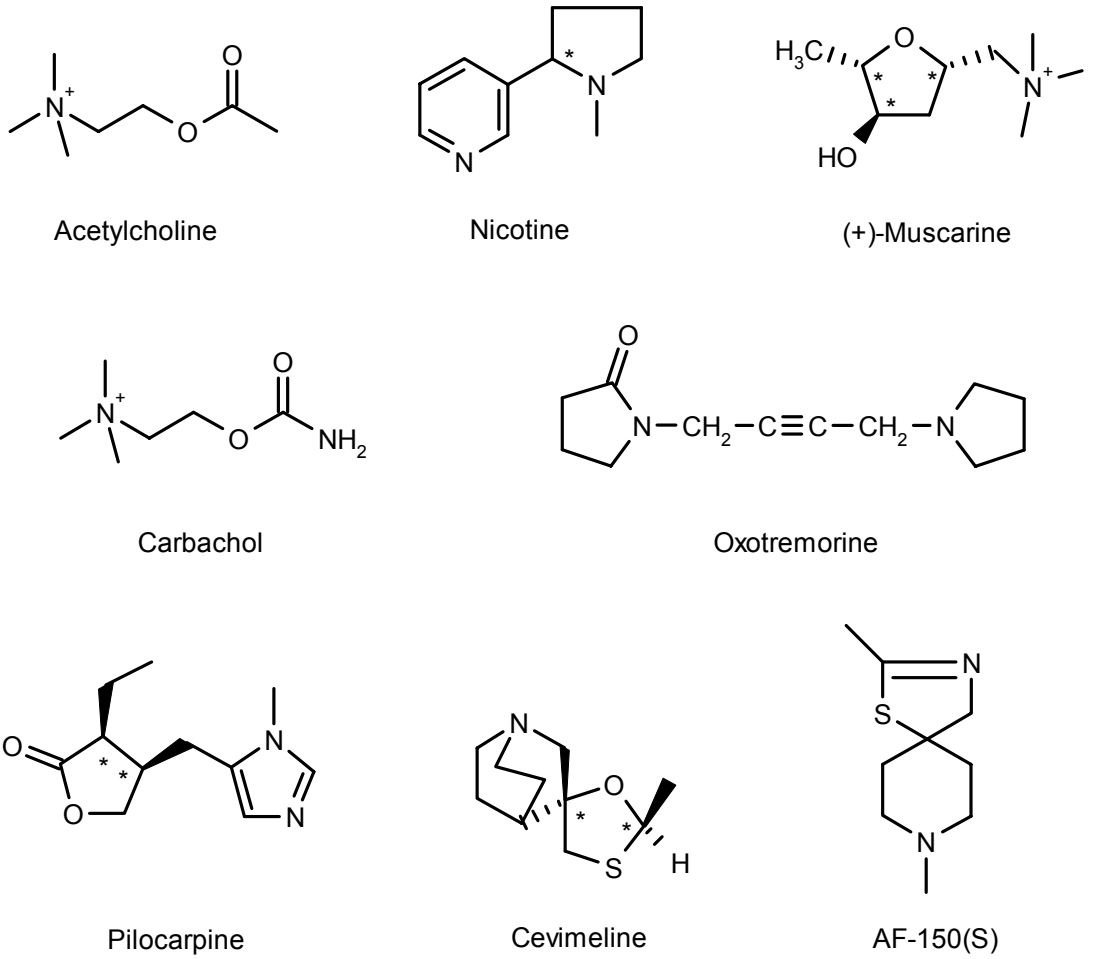


Fig. 1.3 *Chemical structures of selected muscarinic agonists. The asterisks denote the centres of chirality.*

1.1.6.2 Non-selective antagonists

The most prominent representatives within this group are undoubtedly the well known alkaloids atropine and its derivative scopolamine (Fig. 1.4), both highly potent antagonists without any subtype selectivity. The quaternary N-methyl congener of the latter was and is widely used as the radioligand N-methyl-scopolamine (NMS). The formerly considered M_3-selective reference compound 4-DAMP (Fig. 1.4) turned out to be a non-selective compound in binding studies (Dörje et al., 1991).

1.1.6.3 M_1-selective antagonists

With the introduction of pirenzepine (Fig. 1.4; Hammer et al., 1980) the first potent M_1-selective antagonist was described. This compound has low selectivity versus M_4 and good selectivity versus the other subtypes. The derivative guanylpirenzepine (Micheletti et al., 1990) has slightly improved selectivity but decreased affinity. With PD150714, a compound with good selectivity profile (at least 19-fold versus the other subtypes) was developed (Augelli-Szafran et al., 1999). Interesting M_1-selective compounds related to phenglutarimide displaying high stereoselectivity were synthesised (Waelbroeck et al., 1996). Molecular properties of M_1-selective compounds were reviewed (Widzowski et al., 1997).

1.1.6.4 M_2-selective antagonists

With the discovery of the M_4 subtype many formerly considered M_2-selective compounds, e.g. himbacine (Fig. 1.4), were shown to be unable to discriminate between these subtypes which is also true for AF-DX116 (Fig. 1.4) and its congener AF-DX384 (Dörje et al., 1991). Tripitramine was the first truly M_2-selective antagonist with high affinity to M_2 receptors and good selectivity (Melchiorre et al., 1993; Maggio et al., 1994) but displayed non-competitive behaviour in some functional assays. M_2-selective compounds derived from dimethindene were part of this work and are described in detail later. A brain-penetrating M_2 antagonist was reported with BIBN99 (Fig. 1.4) showing low selectivity versus M_4 and good selectivity to the other subtypes (Doods et al., 1993). Recently, a series of reports were given concerning M_2-selective compounds developed by Schering-Plough with high affinity and good or excellent selectivity (Lachowicz et al., 1999; Kozlowski et al., 2000, 2002; Wang et al., 2001,

2002a, b; McCombie et al., 2002; Boyle et al., 2002; Boyle and Lachowicz, 2002), e.g. SCH57790 (Fig. 1.4) and a congener (named “Schering compound” in Fig. 1.4).

1.1.6.5 M_3-selective antagonists

With darifenacin (Fig. 1.5) a compound with M_3-selectivity was developed (Wallis and Napier, 1999). In the last years novel, highly potent compounds with superior selectivity were reported from Banyu Pharm (Banyu Pharm Co Ltd: WO0107406, 2001; Sagara et al., 2002). In an approach using combinatory chemistry, more than 1000 compounds were evaluated regarding their affinity at muscarinic subtypes. An example for a new compound with affinity to M_3 receptors in the subnanomolar range and high selectivity is given in Fig. 1.5 named “Banyu compound”. In recently published studies, follow-up compounds with comparable affinities were presented (Sagara et al., 2003; Ogino et al., 2003).

1.1.6.6 M_4-selective antagonists

The first compound displaying M_4-selectivity was PD102807 (Fig. 1.5) with high affinity to M_4 receptors (Augelli-Szafran et al., 1998). Selectivity was determined to be 186-fold, 77-fold, 37-fold and 486-fold versus M_1, M_2, M_3 and M_5, respectively. Congeners with elongation of the aliphatic methyl group (marked with an arrow in Fig. 1.5) displayed increased affinity and a further improved selectivity profile (Schwarz et al., 2001; Böhme et al., 2002). The propyl-congener was determined to have 1000-fold, 550-fold, 35-fold, and 1000-fold selectivity versus M_1, M_2, M_3 and M_5, respectively. Snake toxins (peptides) taken from mamba venom possess selectivity for muscarinic receptor subtypes, for example MT3 is M_4-selective (for review see Bradley, 2000; Jerusalinsky et al., 2000). However, due to costs and non-competitive kinetics, non-peptide compounds are likely to completely displace these toxins from the laboratories.

1.1.6.7 M_5-selective antagonists

Up to date, no compounds displaying selectivity for this subtype have been reported.

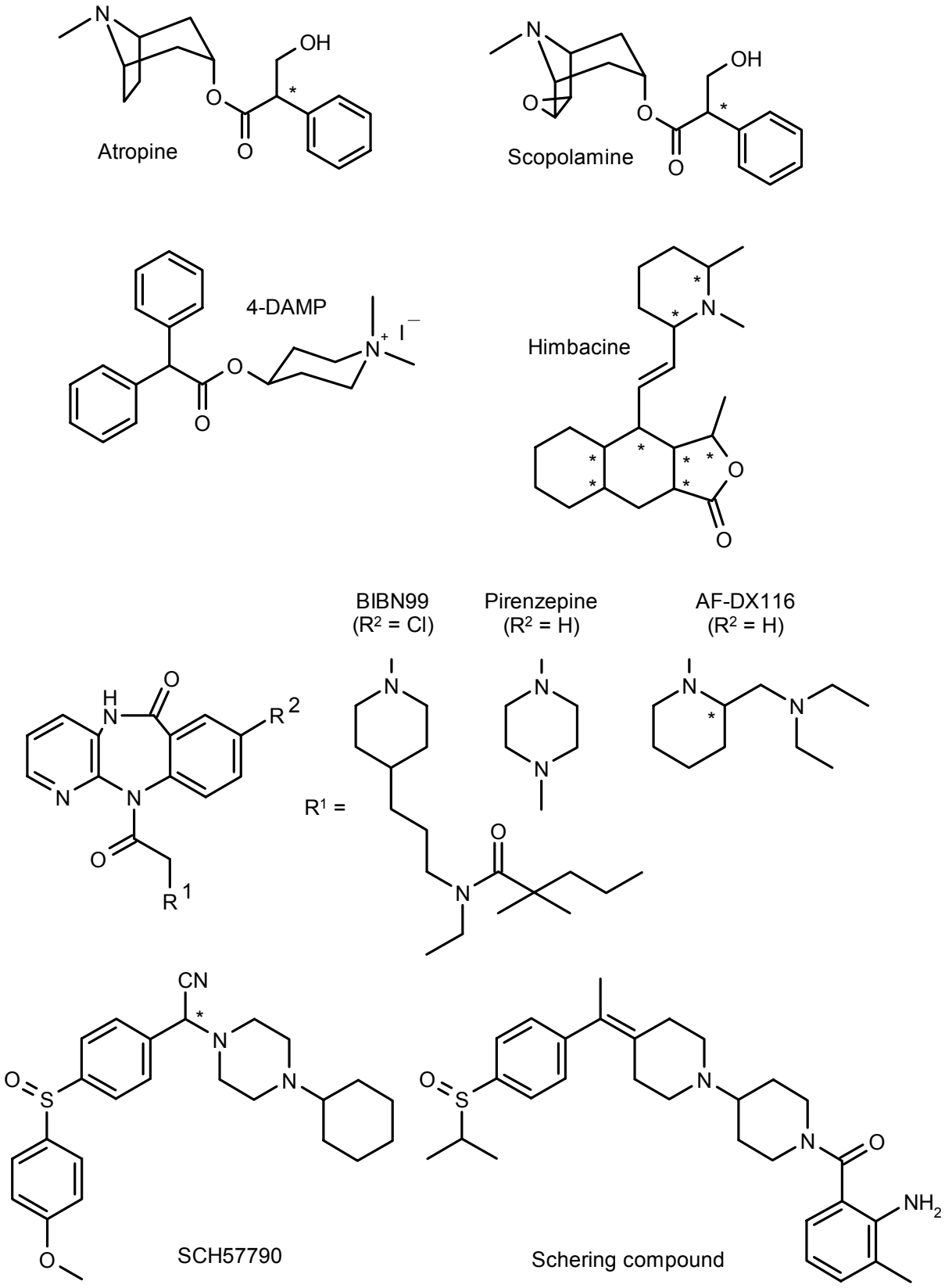
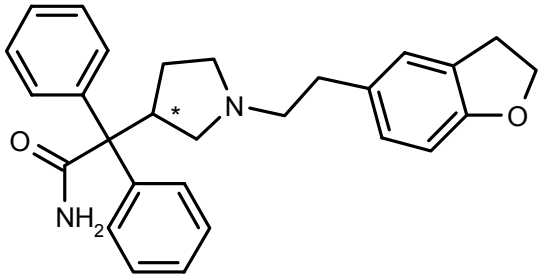
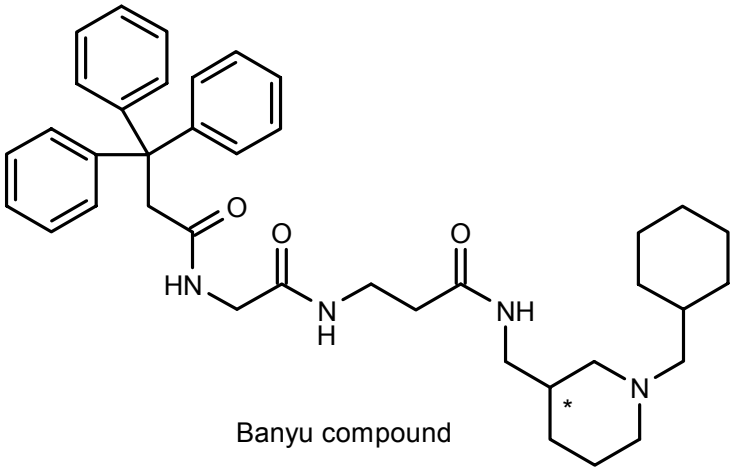


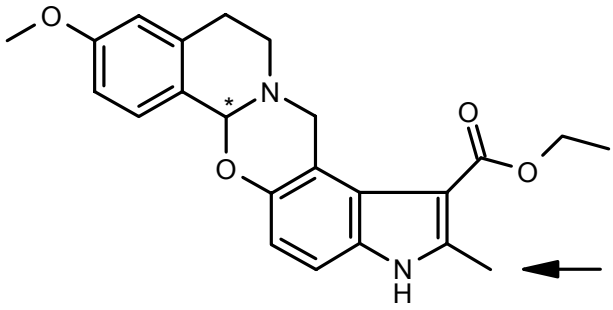
Fig. 1.4 *Chemical structures of selected muscarinic antagonists. The asterisks denote the centres of chirality.*



Darifenacin



Banyu compound



PD102807

Fig. 1.5 *Chemical structures of selected muscarinic antagonists. The asterisks denote the centres of chirality. The arrow marks the modification site leading to highly M_4-selective compounds.*

1.1.7 Therapeutic options

A short summary about the most important therapeutic aspects of muscarinic ligands will be given in the following paragraphs (for review see Eglen et al., 2001; Felder et al., 2000).

1.1.7.1 Peripheral tissues

It is long known, that non-selective, quaternary muscarinic antagonists like N-butyl-scopolaminium bromide can be used as antispasmodics in GIT disorders. Further studies are needed to evaluate, whether M_3-selective compounds like darifenacin are more effective for that purpose (Eglen, 2001). The role of pirenzepine in gastric ulcer therapy (Eglen and Watson, 1996) has decreased with newer, more potent drug classes reaching the market to reduce gastric acid secretion. Muscarinic agonist stimulating M_1/M_3 receptors in salivary glands were shown to be effective in xerostomia in Sjögren's syndrome (see 1.1.6.1). Pilocarpine as a non-selective muscarinic agonist is used in glaucoma therapy to reduce intraocular pressure (IOP) but elicited blurred vision and night blindness as unwanted side effects (both mediated via M_3 receptors). M_3-sparing muscarinic agonists might be better tolerated and highly effective in decreasing IOP since cholinomimetics are the only class of glaucoma drugs facilitating trabecular meshwork outflow (Gil et al., 2001). The use of pirenzepine to reduce development of myopia was suggested and is under investigation (for review see Duncan and Collison, 2003). Ocular application to children was well tolerated (Bartlett et al., 2003). Therapeutic options targeting muscarinic receptors in smooth muscle tissues in the airways and bladder are well known. The use of drugs with prolonged blockade of M_3 receptors in human airways with little effect on M_2 receptors were found to be highly effective bronchodilators, especially in chronic obstrucive pulmonary diseases (COPD). Drug properties important for efficacy in COPD therapy were part of this work and will be discussed later in detail. Anticholingergic drugs are widely used in therapy of unstable bladder to relieve urge incontinence, nocturia and enuresis. The non-selective drugs trospium, oxybutynin and tolterodine are commonly used in therapy (for review see Doggrell, 2001; Yoshimura and Chancellor, 2002), inducing dry mouth as the most important side effect. This side effect was markedly reduced with the introduction of

an once-daily formulation of oxybutynin and tolterodine resulting in lower plasma peak levels (Appell, 2002; Sussman and Garely, 2002). As drug effect within the bladder and side effects in salivary glands are both mediated via M_3 receptors it is of interest to find tissue-specific compounds. Finally, the use of M_2-selective ligands in therapy of arrhythmias was suggested. The M_2-selective antagonist AF-DX116 was used in clinical trials as a chemical pace maker (Schulte et al., 1991), but was discontinued due to retention of cardiologists. M_2-selective agonists were suggested in therapy of tachyarrhythmia (Goyal, 1989). However, up to date this idea has only little relevance.

1.1.7.2 Central nervous system

Studies with KO mice revealed an important role of M_2 and M_4 receptors in mediation of spinal and supraspinal nociceptive processing. M_4-selective muscarinic agonists were suggested to be potential analgesic compounds (Duttaroy et al., 2002) with fewer side effects than it would be expected from the use of M_2 agonists. An interesting approach was reported in a study with mice, using non-selective brain-penetrating muscarinic agonists for analgesia in combination with a quaternary antagonist to block peripheral muscarinic side effects (Tekol and Eminel, 2002).

Influence on dopamine release was detected in KO mice for M_4 and M_5 receptors. In Parkinson's disease (PD) a proceeding loss of dopaminergic neurons within the substantia nigra and a reciprocal increase in cholinergic transmission in the striatum was detected (for review see Centonze et al., 1999) leading to disorders of the extrapyramidal system. At present, muscarinic antagonists with high affinity for M_1 and M_4 receptors, like biperiden, are in therapeutic use (Grimm et al., 1994). Selective, brain-penetrating M_4 antagonists (see also 1.1.6.6) were suggested to be a novel therapeutic approach in PD therapy (Salamone et al., 2001; Mayorga et al., 1999). M_1/M_4 agonists were suggested to be helpful in therapy of psychosis (Felder et al., 2001). Additionally, an involvement in dopaminergic transmission in rewarding effects following drug abuse was determined for M_5 receptors (Forster et al., 2001). Mice lacking this subtype had attenuated morphine withdrawal symptoms but unchanged analgesia (Basile et al., 2002). In Asian countries good results were achieved in detoxification of heroin addiction with scopolamine. Hence, it was

suggested that this was possibly a M_5-mediated effect and M_5 antagonists might serve as a new approach in the treatment of opiate addiction (Yang, 2002).

Handling of Alzheimer's disease (AD) (see also 1.1.6.1 and 1.1.6.4) is one of the most difficult tasks to public health in our times. The histopathologic hallmarks of AD are neurofibrillary tangles consisting of hyperphosphorylated tau protein and β-amyloid plaques (for review see Selkoe, 2001). Early reports on decreased activity of cholinergic markers in the brains of AD patients (Davies and Maloney, 1976) and the finding that scopolamine reduced perfomance in memory tasks (Drachman and Leavitt, 1974) led to the cholinergic hypothesis in AD (Bartus et al., 1982). This hypothesis was supported with the therapeutic success of currently used AChE inhibitors. However, AChE inhibitors provide a merely symptomatic therapy with little efficacy. Several new therapeutic aspects were and still are under investigations (for review see Kumari and Ram, 2001; Dominguez and De Strooper, 2002; Yamada and Toshitaka, 2002) but in the following focus is laid on the muscarinic system. Two approaches are currently examined. Firstly, the application of M_1-selective agonists (see also 1.1.6.1) as disease modifying drugs with influence on β-amyloid metabolism and tau hyperphosphorylation (for review see Fisher, 2000, 2002). However, up to now, all clinical studies with so-called "M_1 agonists" showed little or no positive effects in clinical trials, perhaps due to low selectivity or inappropriate kinetic properties of the tested drugs. There is a strong need for M_1-selective agonists with improved drug characteristics to clarify the utility of M_1 agonists in AD. The second approach is, like AChE inhibitors, a non disease modifying approach. Inhibitory M_2 autoreceptors were shown to control ACh release in several brain regions (Feuerstein et al., 1992). Therefore, it was suggested that M_2 antagonists might improve cognition. Indeed, blocking these autoreceptors with selective M_2 antagonists was shown to facilitate ACh release and improve cognitive performance (Packard et al., 1990; Quirion et al., 1995). However, the use of M_2 antagonists in AD might be limited because the number of cholinergic neurons decreases over time in AD, reducing the amount of available prejunctionally located target sites (Sheardown, 2002). Further clinical studies with brain-penetrating, highly selective M_2 antagonists are needed to clarify this issue.

1.1.8 Diagnostic potential

With positron emission tomography (PET) a medical imaging technique with high potential is becoming more and more popular. PET studies can provide *in vivo* information about receptor density and blood flow in a special tissue (Bartenstein, 2002). PET ligands for receptor imaging should provide a high affinity to the target site, high selectivity and specificity. At present, diagnosis in AD is made post-mortem. With PET tracers selective for M_2 receptors (which decrease in AD) the evaluation of new disease modifying drug therapies could be imaged *in vivo*. Further, a diagnosis could be posed before AD becomes clinically relevant (Volkow et al., 2001; Silverman and Small, 2002; Petrella et al., 2003).

1.2 Histamine receptors

Already before world war I, first reports on the physiological actions of histamine were reported (Dale and Laidlaw, 1910). In the following decades pharmacologically different histamine receptor subtypes were identified. In 1997 the IUPHAR differentiated three different GPCR belonging to the histaminergic family, named H_1, H_2 and H_3 receptors (Hill et al., 1997). First cloning of the H_2 subtype positively coupled to adenylyl cyclase (AC) via G_s was reported in the early 1990's (Bakker et al., 2000). At that time, bovine and later human H_1 receptors were cloned, too (Yamashita et al., 1991; De Backer et al., 1993). Stable expression of this subtype in CHO cells clearly demonstrated its coupling with G_q, increasing intracellular Ca^{2+} level (Moguilevsky et al., 1994; Smit et al., 1996). The hH_1 receptor gene was cloned and completely mapped to chromosome 3, identified as an intronless gene (Fukui et al., 1994; Bakker et al., 2000). In the late 1990's the hH_3 receptor was cloned for the first time and later on the gene was mapped (Lovenberg et al., 1999; Wiedemann et al., 2002). This subtype is negatively coupled to AC via $G_{i/o}$. In the years 2000 and 2001, several groups published in close succession the cloning and characterisation of a previously unknown histamine receptor, denominated H_4 (Nakamura et al., 2000; Oda and Matsumoto, 2001; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). This newly identified subtype is closely related to the H_3 subtype and couples via $G_{i/o}$ to AC. Recently, a histamine-gated chloride channel was identified in *Drosophila* (Lopez, 2002). Thus, the search for previously undetected ligand-gated ion channels

sensitive to histamine started in humans. Fig. 1.6 gives a phylogenetic tree between some members of the GPCR family. A close relationship between H_1 and muscarinic receptor subtypes can be seen. Greatest amounts of histamine within the human body are located in tissue mast cells and enterochromaffin-like cells in the GIT. To a smaller extend histamine is stored in basophil leukocytes and platelets.

In the following paragraphs a brief summary of the most important findings concerning the H_1 receptor is given.

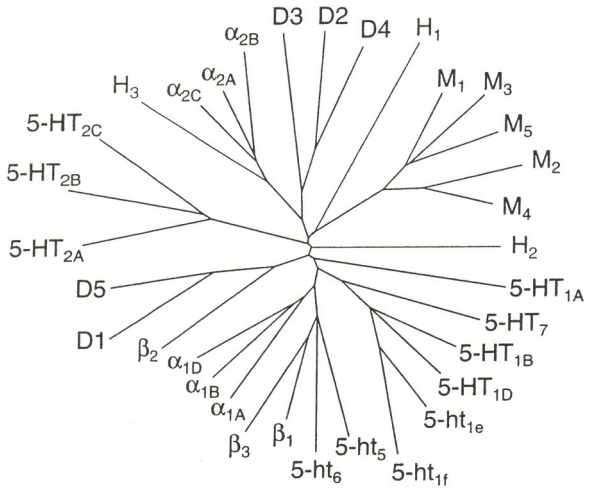


Fig. 1.6 *Phylogenetic tree of homology between members of the GPCR family of amine transmitter systems. Taken from Leurs et al., 2000.*

1.2.1 H_1-receptor distribution and function

H_1 receptors are widely distributed within the human body (for review see Hill et al., 1997). In vascular endothelial cells, H_1 receptor stimulation leads to increased vascular permeability as a result of endothelial cell contraction. A variety of transmitter molecules and proteins can be released from endothelial cells, including the vasodilator NO. Influence of histamine on the cardiovascular system is complex and dose-dependent. Application of histamine results in a decrease in blood pressure as a result of endothelial NO liberation. Several smooth muscles, especially in human airways and GIT, contract as a result of H_1 stimulation. In the adrenal medulla, H_1 receptor stimulation facilitates the release of adrenaline and noradrenaline. H_1 receptors are also widely distributed within the brain of humans and other species.

1.2.2 Ligands at H_1 receptors

In this paragraph focus is laid on antagonists. The molecular properties of agonists and antagonists at H_1 receptors were extensively reviewed (Leurs et al., 1991, 1995; Hill et al., 1997). So-called “first generation” H_1 antagonist like diphenhyramine (Fig. 1.7) were penetrating into the CNS where they elicited sedation as a side effect. This effect was clearly confirmed as an H_1 effect in human studies using dimethindene (Nicholson et al., 1991). As a consequence, new molecules were designed with less lipophilic structures. Introduction of hydrophilic moieties in the molecular structures of already known H_1 antagonists resulted in less lipophilic drugs with comparably low brain penetration, e.g. hydroxyzine → cetirizine (Fig. 1.7) or azatidine → loratadine (Fig. 1.7) (Leurs et al., 1995). These compounds are generally considered as non-sedating “second generation” H_1 antihistaminergic drugs with fewer CNS side effects. Some tricyclic antidepressant drugs like doxepin (Fig. 1.7) display high affinity to this receptor next to many other receptor sites (Figge et al., 1979). Chemical diversity within the vast amount of known H_1 antagonists is large, especially within the newer compounds. Up to now, no model exists that is capable of fitting all of them to an interaction with the H_1 subtype. Mepyramine (= pyrilamine; Fig. 1.7) was discovered almost half a century ago and is still the most popular radioligand for affinity determinations at H_1 receptors (Marshall, 1955). It is also noteworthy, that many previously described H_1 antagonists were found to be inverse agonists (Leurs et al., 2002). Compounds derived of the well known H_1 antagonist dimethindene were part of this work. Therefore more details about dimethindene are reported later in the Aims chapter (see 2.7).

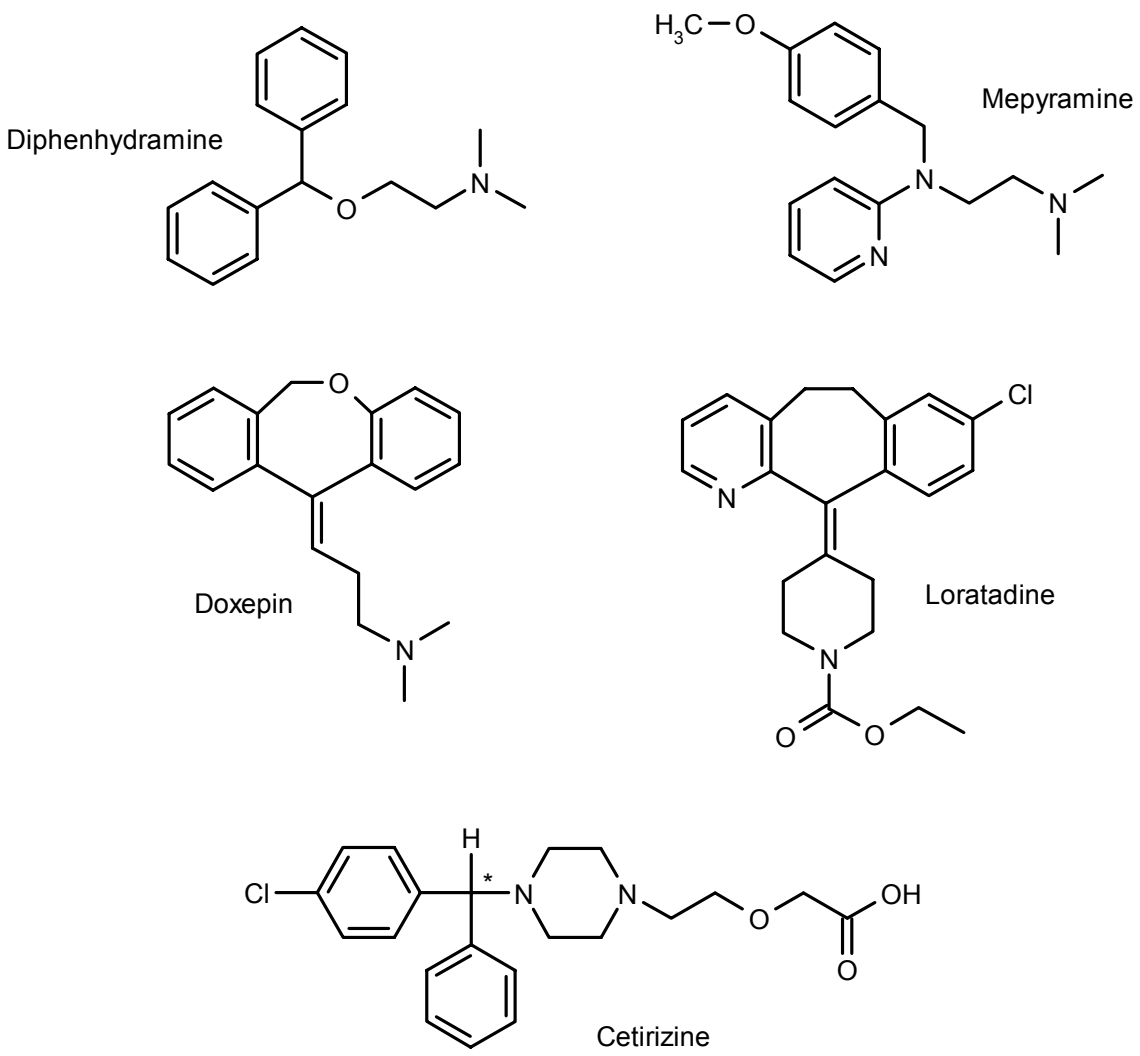


Fig. 1.7 *Chemical structure of selected H_1 antagonists. The asterisk denotes the centre of chirality.*

1.2.3 Therapeutic implications

The most important therapeutic use of H_1 antagonists are allergic diseases, such as rhinoconjunctivitis, urticaria and atopic dermatitis (Baroody and Naclerio, 2000). "Second generation" H_1-antihistamines are widely used for that purpose with good efficacy and little side effects. However, studies concerning the use of this class of drugs in asthma were disappointing (Mincarini et al., 2001). The use of antihistamines as analgesic drugs provided conflicting data and needs further evaluation (Raffa, 2001). "First generation" H_1 antagonists are still in use as mild sedatives and for prevention of motion-sickness.

1.3 Serotonin receptors

Serotonin (5-hydroxytryptamine, 5-HT) has a long history as an endogenous signalling molecule in humans and animals mediating a great variety of physiological effects. Early studies were carried out in guinea-pig ileum (GPI) to clarify the role of this newly described agent (Rocha e Silva et al., 1953). In the late 1950's two different receptors for 5-HT were identified in GPI (Gaddum and Picarelli, 1957). They were named M- and D-receptor, because the responses at these subtypes could be blocked with **m**orphine and **d**ibenzyline. It was in the late 1980's and the begin of the 1990's when a burst in serotonin receptor pharmacology took place with cloning techniques coming up. An almost incredible amount of 5-HT receptor subtypes were cloned in humans and several animals. NC-IUPHAR for serotonergic receptors confirmed already seven different receptor families for serotonin in 1994 (Hoyer et al., 1994). At this time, it seems as if 5-HT plays a unique role in the monoamine neurotransmitter family with regard to receptor diversity. With posttranslational modifications, alternative splicing, oligomerization and heteromerization an almost endless complexity among this receptor class seems possible. The former D-receptor is now called 5-HT_2, the M-type 5-HT_3 receptor. Fig. 1.8 gives an overview of the actual findings on serotonergic receptors. It is important to note that all identified subtypes belong to the class of 7-TM GPCR-family with exception of the 5-HT_3 receptor which was identified as an ligand-gated ion channel.

Serotonergic receptors are widely distributed throughout the periphery and the central nervous system of the human body. The vast amount of existing data concerning all known subtypes was extensively reviewed during the last years (Hoyer et al., 1994, 2002; Barnes and Sharp, 1999). As it is impossible to address all subtypes in this work, focus was laid on the 5-HT_3 and 5-HT_4 receptor subtypes which were subjects of research in this dissertation. It is interesting to notice, that 95% of the total 5-HT in the human body is found in the GIT. 90% of that amount are located in enterochromaffin cells and 10% in enteric neurons. The remainder of 5-HT (5%) is found in the brain. Serontonergic neurons constitute about 2% of all myenteric neurons in the GIT. Virtually all of the 5-HT in blood is derived from GIT, where it is released from the bowel following stimulation. 5-HT is additionally a constituent of platelets and is participating in aggregation and coagulation of blood (Kim and Camilleri, 2000).

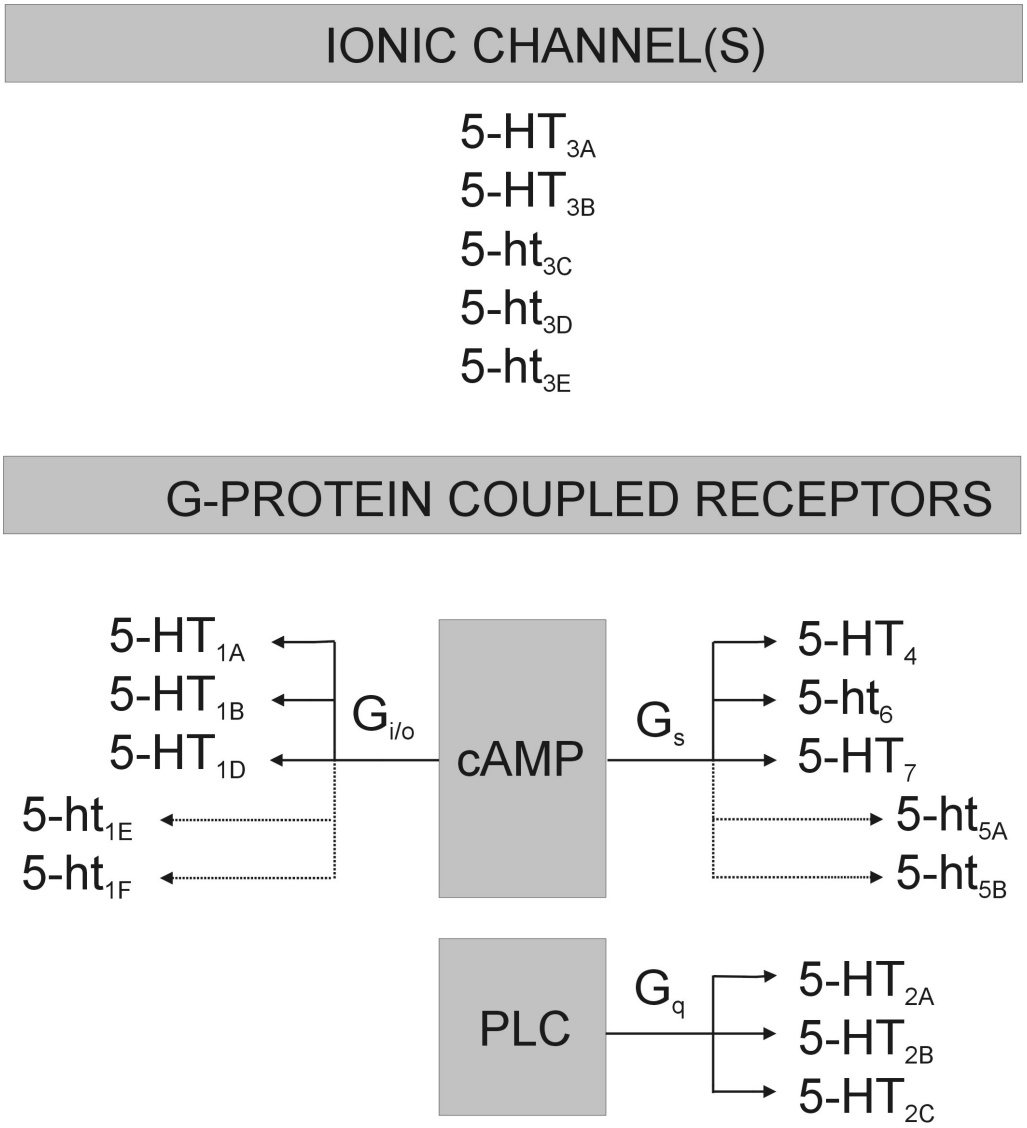


Fig. 1.8 *Summary of the current classification of 5-HT receptors (modified from Hoyer et al., 2002). Subtypes for which up to now no physiological function was demonstrated are given in uncapitalised letters.*

1.3.1 5-HT_3 receptors

5-HT_3 receptors were identified as ligand-gated ion channels. First reports were given in tissue preparations of guinea-pig submucosus plexus with patch clamp experiments (Derkach et al., 1989). This finding was supported with the first cloning of a 5-HT_3 receptor from NCB20 cells (mouse neuroblastoma x chinese hamster embryonic brain cells) and expression in xenopus oocytes resulting in a serotonin

sensitive increase in conductance (Maricq et al., 1991). The formed ion channel was found to be non-selective, as opening resulted in Na^+, Ca^{2+} influx and K^+ efflux (Davies et al., 1999). The ion-channel was suggested to be composed of five subunits with regard to the related nACh receptor family. The ultrastructure of 5-HT_3 receptors could be elucidated with purified receptors derived from NG108-15 cells (mouse neuroblastoma x rat glioma cells) and mouse tissue (Boess et al., 1995; Green et al., 1995). Fig. 1.9 gives a computer filtered picture of purified 5-HT_3 receptors. The pentameric structure was confirmed in these studies. Receptors were modelled as cylinders 11 nm in length, 8nm in diameter and with a central cavity of 3 nm in diameter. For each of these five subunits a 4TM structure was proposed based on hydropathy profiles and homology to nACh receptors (Hovius et al., 1998). Direct evidence confirming this theory was derived with a set of antibodies recognising different structural domains of 5-HT_3 receptors. (Spier and Lummis, 2002). With the mapping of the h5-HT_{3A} gene (located on chromosome 11) a detailed topological model of the receptor, given in Fig. 1.10, was possible (Brüss et al., 2000b). The coding region of the h5-HT_{3A} gene consists of 9 small exons separated by relatively large introns and stretches over a total of 15 kilobases. Evidence was given that the TM2 domains of all subunits account for the formation of the ion pore (Panicker et al., 2002) and that the large second intracellular loop (i2) is in parts responsible for the single channel conductance (Kelley et al., 2003). Receptor purification studies (McKernan et al., 1990) and electrophysiological studies (Hussy et al., 1994) gave strong evidence that additional subunits next to 5-HT_{3A} were abundant. Additional 5-HT_{3B} (Dubin et al., 1999; Davies et al., 1999), 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits were found after extensive search, recently (Dubin et al., 2001; Niesler et al., 2003). Apart from heteroassembly between the 5-HT_{3A} and 5-HT_{3B} subunits to functional channels (in contrast to 5-HT_{3B} alone) at the cell surface, little is known about the functional importance of these subunits (Boyd et al., 2002).

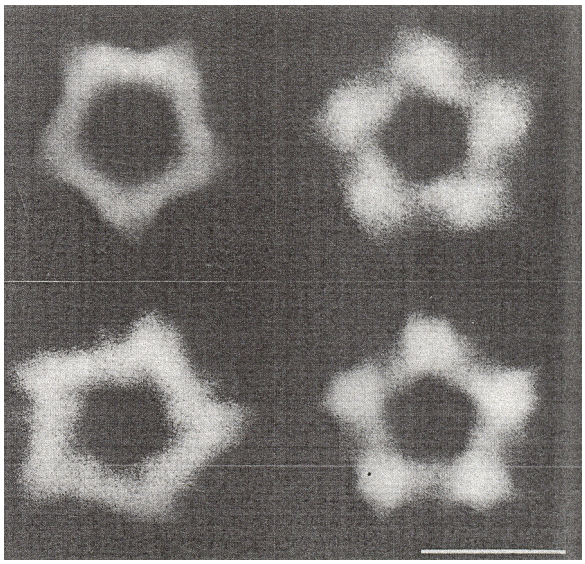


Fig. 1.9 *Computer filtered image of purified 5-HT$_3$ receptors from NG108-15 cells. Bar = 5 nm. Taken from Boess et al., 1995.*

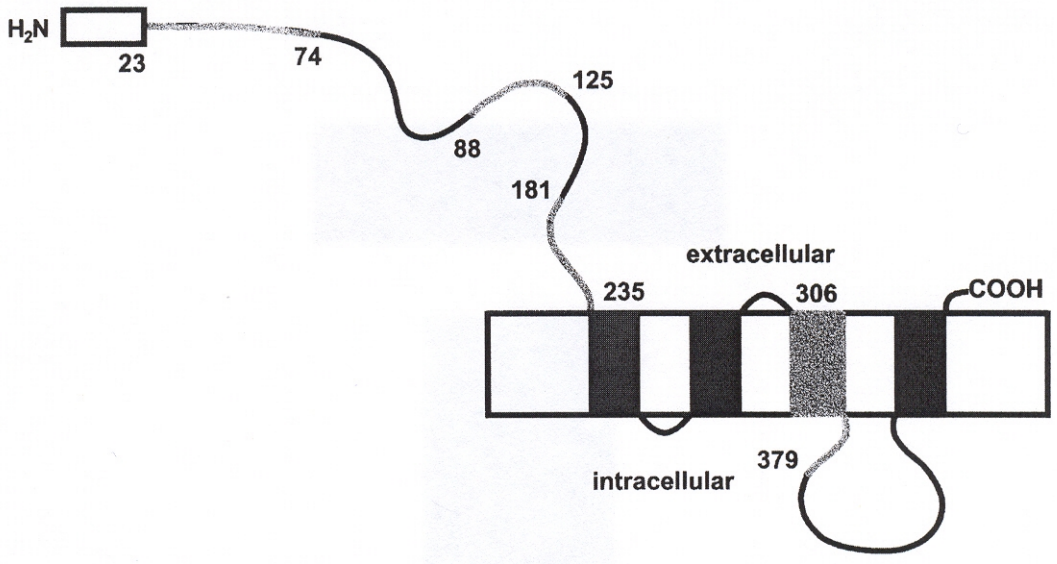


Fig. 1.10 *Topological model of the h5-HT$_{3A}$ receptor protein with respect to particular exons. TM domains were localised with hydrophobicity analysis. The particular exons are alternatively shown in grey and black. The numbers indicate the amino acid residues at exon-intron boundaries. The black box (N-terminus) shows the 23 residues long signal sequence. Taken from Brüss et al., 2000b.*

1.3.2 5-HT_3-receptor distribution

5-HT_3 receptors are exclusively located on neurons of central and peripheral origin mediating rapid depolarisation. The response following activation desensitises and resensitises quickly (Hoyer et al., 1994). CNS distribution of 5-HT_3 receptors in several species was extensively examined in binding studies (Kilpatrick et al., 1987; Fletcher and Barnes, 1999; Miller et al., 1992). Considerable concentrations of 5-HT_3 binding sites were detected in the raphe nuclei (located in midbrain, pons and medulla oblongata), where the major part of serotonergic cell bodies is located (Molderings, 2002). These neurons project in various other brain regions. Lower concentrations were detected in other brain regions. Interestingly, highest amounts of 5-HT_3 sites were identified in the area postrema of all examined species including humans. However, apart from this region, amount and distribution pattern differ between species (Marazziti et al., 2001; Kilpatrick et al., 1989). In the periphery, 5-HT_3 receptors are located on pre- and postganglionic autonomic neurons, on neurons of the sensory nervous system, and on intrinsic neurons of the GIT (Hoyer et al., 2002).

1.3.3 Ligands at 5-HT_3 receptors

Based on early findings in 1953 that cocaine (Fig. 1.11) acted as a M-type (later named 5-HT_3) antagonist (Gaddum and Picarelli, 1957), J. R. Fozard started a screening for novel 5-HT_3 antagonists. Based on variation of the local anaesthetic molecule procaine, metoclopramide (MCP) (Fig. 1.11) was identified as a potent but non-selective compound (Fozard and Mobarok Ali, 1978). In a series of tropane esters, MDL72222 (Fig. 1.11) was found as the first potent and selective compound (Fozard et al., 1979; Fozard, 1984). At that time, tropisetron (Fig. 1.12) was synthesised in the laboratories of Sandoz (Richardson et al., 1985) and Glaxo published first data on ondansetron (Stables et al., 1987; Butler et al., 1988), both compounds being highly potent 5-HT_3 antagonists with good subtype selectivity, later approved to the market. In the following years GR65630 (Fig. 1.11) was identified. This compound was not a milestone as a therapeutic drug but its tritiated form served and still serves as an important tool in radioligand binding studies (Kilpatrick et al., 1987).

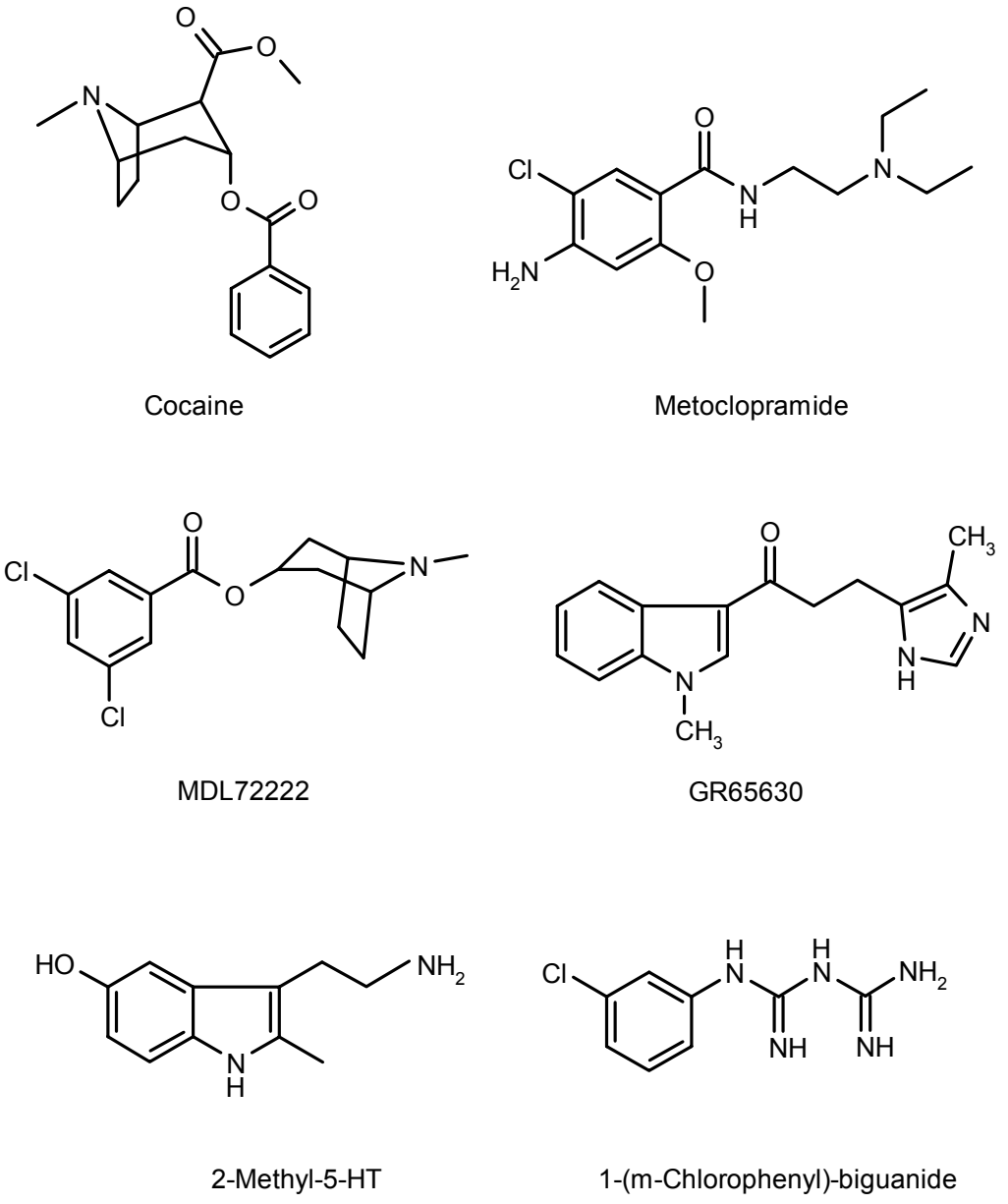


Fig. 1.11 *Chemical structures of important ligands at 5-HT$_3$ receptors.*

Later on, granisetron (Sanger and Nelson, 1989), dolasetron (Boeijinga et al., 1992) and finally alosetron (Clayton et al., 1999) were approved to the market (Fig. 1.12). Alosetron was removed from the market (Charatan, 2000) because several deaths were reported due to ischaemic colitis as a severe side effect. Recently, this drug was reintroduced with stern obligation for therapy of diarrhoea predominating irritable bowel syndrome in women. It is obvious that all approved 5-HT$_3$ antagonists (Fig. 1.12) are closely related to the physiological ligand serotonin.

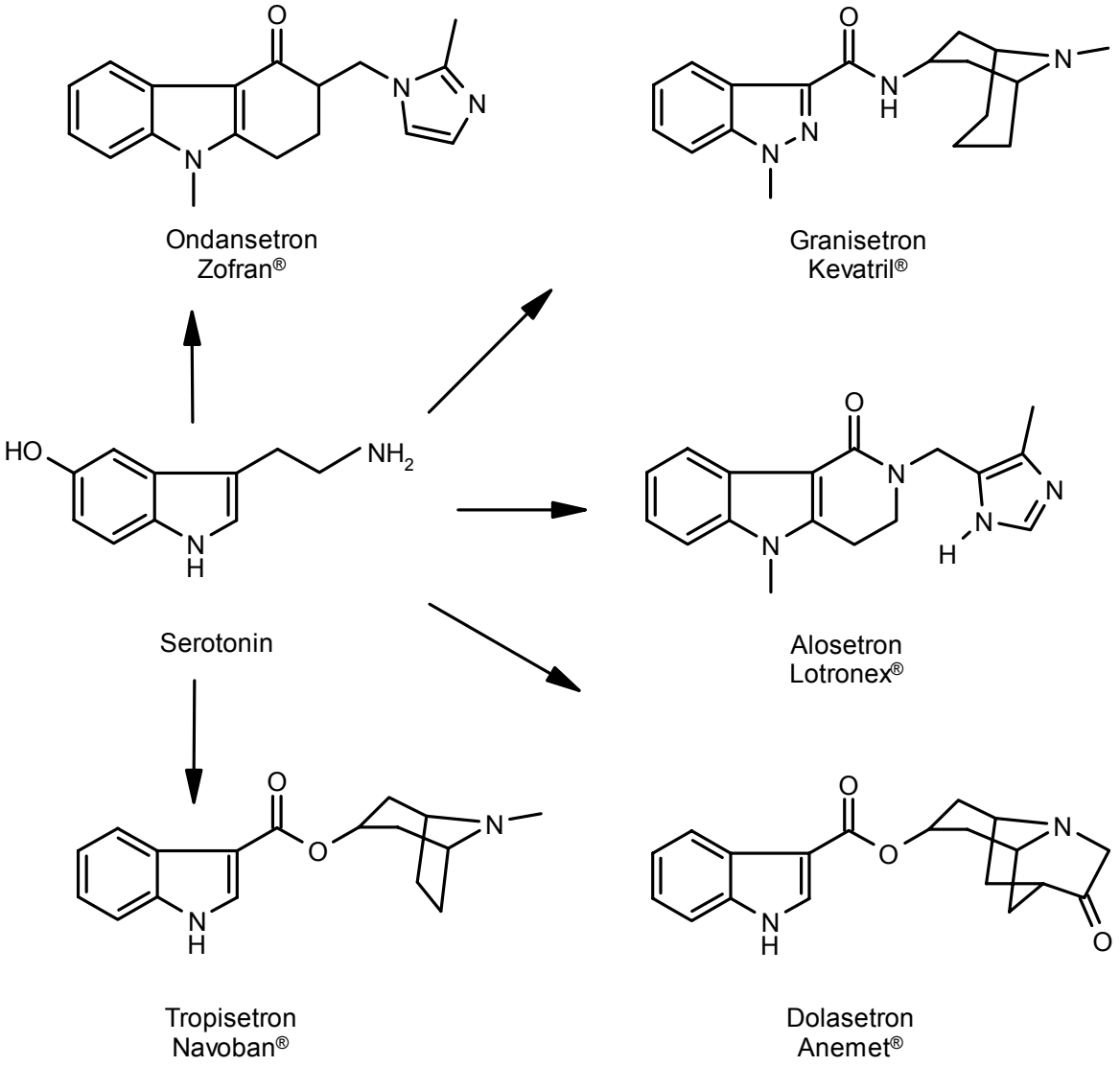


Fig. 1.12 *Approved 5-HT$_3$ antagonists with their brand names currently used in Germany in comparison to the endogenous receptor ligand serotonin.*

Comparably little has been reported about 5-HT$_3$ agonists. The non-selective and metabolic unstable derivative 2-methyl-5-HT (Fig. 1.11) was identified as an agonist. Actually the most potent available partial agonist at 5-HT$_3$ receptors is 1-(m-chlorophenyl)-biguanide with good subtype selectivity, displayed in Fig. 1.11 (Kilpatrick et al., 1990b).

1.3.4 Functional models

In GPI contraction is elicided by 5-HT via stimulation of cholinergic neurons in the plexus myentericus. In rabbit isolated heart, 5-HT can modulate input to the heart via sympathetic and parasympathetic neurons. In the presence of muscarinic antagonists positive chronotropic and intropic effects are elicited by 5-HT (indirectly via noradrenaline). Negative chronotropic and intropic effects are seen in the presence of ß-blockade (via acetylcholine). However, these two models provide only indirect 5-HT_3 effects. As a direct effect of 5-HT at 5-HT_3 receptors the depolarisation of rat vagus nerve (RVN) can be measured (Kilpatrick et al., 1990a; Oxford et al., 1992).

The most important *in vivo* model is the vagally mediated reflexive fall in heart rate and blood pressure (von Bezold-Jarisch reflex) following an i.v. bolus application of 5-HT, usually measured in anaesthetised cat or rat, believed to be mediated through depolarisation of afferent nerve endings in the right ventricle (Fozard, 1984).

1.3.5 Therapeutic options

A brief overview concerning the therapeutic potential of 5-HT_3 ligands is given in the following paragraphs. The use in GIT disorders will be presented in the 5-HT_4 chapter (see 1.3.10.4).

1.3.5.1 Antiemetic properties

Cancer therapy was revolutionised with the introduction of 5-HT_3 antagonists. These drugs potently inhibit acute emesis (onset during the first 3 h) following chemo- and radiotherapy (Günther, 2002). These quite aggressive therapy regimen trigger the liberation of 5-HT from enterochromaffin cells in the GIT. 5-HT activates 5-HT_3 receptors located on afferent vagal fibres and in the area postrema resulting in nausea and vomiting. The area postrema was identified as an important area for control of nausea and emesis. However, little efficacy was achieved with 5-HT_3 antagonists in therapy of delayed emesis (persisting 3-5 days). Actually steroids in combination with MCP are in use to prevent delayed emesis. Recently, good results were obtained with additional application of NK_1 antagonists (de Wit et al., 2003).

1.3.5.2 Use in mental disorders

The use of drugs with affinity to 5-HT_3 receptors in mental disorders was extensively reviewed (Silverstone and Greenshaw, 1996; Greenshaw and Silverstone, 1997; Barnes and Sharp, 1999). The potential use of 5-HT_3 antagonists in anxiety and psychotic disorders was investigated in several animal models. Some studies yielded good preclinical data, even though sometimes conflicting results were obtained. However, up to now, no double-blind clinical study showed positive results in humans, making an effective use of this drug class in anxiety and psychotic disorders unlikely. As findings came up that noradrenaline release was facilitated by 5-HT_3 receptors, the question of a potential use of 5-HT_3 agonists as antidepressant drugs came up. With SR57227A (Bachy et al., 1993) a potent, brain-penetrating agonist was described. This compound showed positive effects in some animal models. Further studies are needed to confirm the utility of 5-HT_3 agonists as antidepressant drugs in humans. Conflicting results were also reported in several studies concerning the use of 5-HT_3 antagonist in drug abuse, depending on the investigated drug. Slightly reduced ethanol intake was, for example, seen in animals and humans, but up to now no convincing study reports on the utility of 5-HT_3 antagonists in drug abuse syndromes were published. With the discovery that 5-HT_3 receptors influence ACh release in several brain regions, interest came up for a use in AD. First studies with alosetron in healthy humans gave positive results (Preston et al., 1991), together with the encouraging finding that 5-HT_3 receptor density is unchanged in the brains of AD patients (Barnes et al., 1990). Further studies are needed to confirm the efficacy of 5-HT_3 antagonists in AD.

In summary, most clinical trials following the extensive research in animal models for new therapeutic indications were disappointing. Little hope is left to find an effective use for 5-HT_3 ligands in the most mental disorders discussed above. In contrast to animal studies, which often gave rise to hope, the findings in human clinical trials lead to the question, whether some of the used animal models are suitable to mirror conditions found in humans.

1.3.5.3 Antinociceptive effects

5-HT_3 receptors involved in nociception were found on sensory afferent neurons and in the dorsal horn of the spinal cord (Hamon et al., 1989). Evidence for participation

in cardiac pain was given. The most interesting findings were reported for the use of tropisetron in rheumatic diseases. Local injections resulted in a pronounced analgesic effect in various diseases of the locomotor system (such as tendinopathies and peri-arthropathies) comparable to the effect achieved with injection of dexamethason plus lidocaine, but with prolonged time of action (Stratz and Müller, 2003; Müller and Stratz, 2003). Beside the antinociceptive effects, antiphlogistic actions contributed to the high efficacy in this study. The detailed mechanisms of action remain yet to be elucidated.

1.3.6 5-HT_4 receptors

First reports on the 5-HT_4 receptor subtype were given in the late 1980's (Dumuis et al., 1988). Studies at mouse embryo colliculi neurons and guinea-pig brains revealed the existence of a previously unknown receptor subtype belonging to the serotonergic family. This subtype was the first 5-HT receptor to be identified with positive coupling to adenylate cyclase, elevating intracellular cAMP level. In the following decade different splice variants of this subtype were cloned from several species. Comparably late the human 5-HT_4 receptor was cloned (Van den Wyngaert et al., 1997). Mapping of the human 5-HT_4 receptor gene (Bender et al., 2000) pinpointed coding regions for all previously known splice variant. The coding sequence for the first nine amino acids of the 5-HT_4 receptor protein was not allocated and is likely to be found on an additional exon located upstream exon 2. Fig. 1.13 gives a schematic graph of the human 5-HT_4 receptor gene structure with the possible splicing sites, whereas Fig. 1.14 shows a model of the resulting receptor protein. All splice variants except 5-$HT_{4(h)}$ are identical up to amino acid 358 and differ only in the C-terminus. 5-$HT_{4(h)}$ displays an insertion of 14 amino acids in the second extracellular loop and can be combined with all possible splice variants in the C-terminus. The physiological role of the various splice variants is not completely understood and will be dealed with in the Discussion chapter.

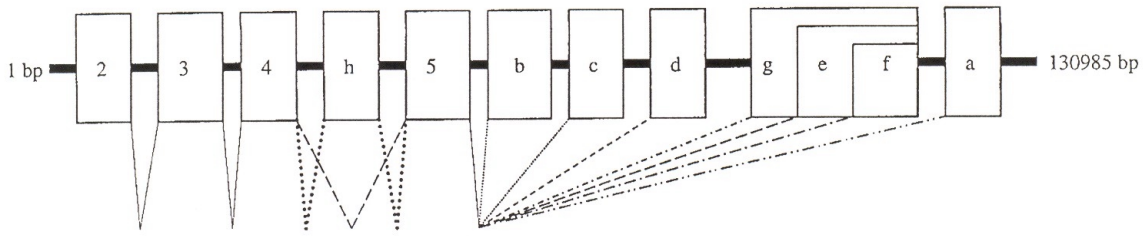


Fig. 1.13 *Alternative splicing possibilities among the different 5-HT$_4$ receptor exons are indicated by connection lines. Boxes represent exons, whereas introns are shown as bold lines. The dotted line between exon 4 and 5 represents the splicing that will include exon h into the mRNA, whereas the splice event following the dashed lines omits exon h. Downstream exon 5 different splices are depicted by differently formatted lines. Modified from Bender et al., 2000.*

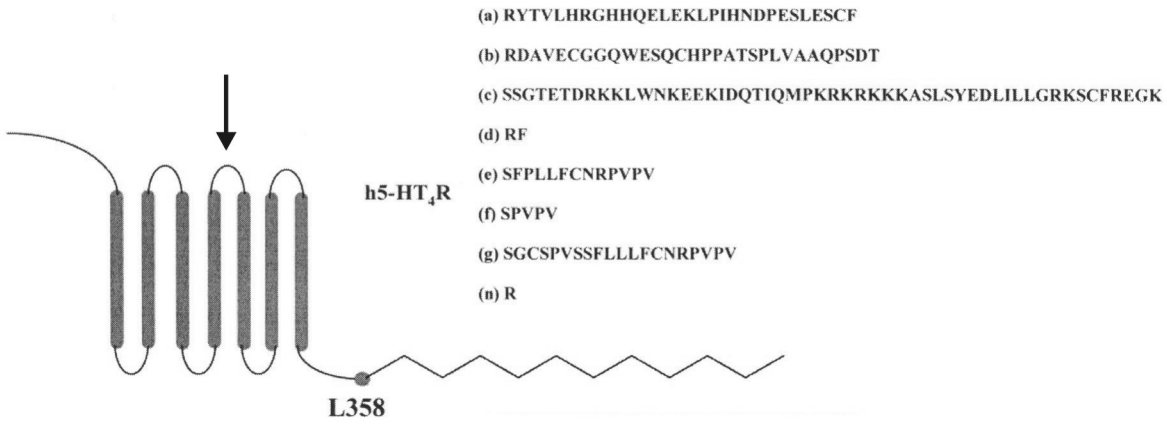


Fig. 1.14 *Schematic model of the 5-HT$_4$ receptor protein with 7TM domains. C-terminal amino acid sequences of the human splice variants (a-g, n). The arrow indicates the site for a 14 amino acid insertion in the second extracellular domain of the 5-HT$_{4(h)}$ splice variant. Modified from Langlois and Fischmeister, 2003.*

1.3.7 5-HT$_4$-receptor distribution

5-HT$_4$ receptors were reported to be widely distributed in the CNS and periphery (for review see Eglen et al., 1995; Hegde and Eglen, 1996). Autoradiography studies in several species (Waeber et al., 1994; Reynolds et al., 1995; Patel et al., 1995) revealed within the CNS highest 5-HT$_4$ concentrations on neuronal structures in the limbic system (that is, e.g., the frontal cortex, hippocampus and amygdala) and lower

concentrations in several other brain regions. With regard to the periphery, 5-HT_4 receptors were detected in various organs (Fig. 1.15). In hearts of human and pig 5-HT_4 receptors were detected on muscle cells of atria and sinu-atrial node (Kaumann, 1990). 5-HT_4 density in human heart was low in comparison to ß-receptors. The calculated ratios were approximately 1 : 5 : 10 for 5-HT_4 : $ß_2$: $ß_1$ receptors (Kaumann et al., 1996). The 5-HT_4 subtype was detected in lower urinary tract of human and other species facilitating ACh release (Tonini and Candura, 1996). Only little influence on vasculature was reported for 5-HT_4 receptors, for example, in some blood vessels in sheep. Reports were given that the secretion of corticosteroids and aldosterone from the adrenal cortex in frogs and humans is promoted by 5-HT_4 (Idres et al., 1991). Finally, 5-HT_4 receptors were detected in the GIT (Craig and Clarke, 1990). The GIT is one of the most complex regions in the human body containing approximately 10^8 intrinsic neurons. At least 25 different neurotransmitter were detected within the enteric nervous system (Molderings, 2002). This highly complex physiological network is currently far from being completely understood. 5-HT_4 receptors are located on mucosal cells involved in secretory functions and on smooth muscle cells of the longitudinal and circular muscles. Additionally, 5-HT_4 receptors are located on neuronal structures.

Several other serotonergic receptor subtypes were identified in the GIT. Serotonin is considered to be a key player in GIT with major influence on peristaltic reflex, secretion and sensory fibres termed as “mediator of the brain-gut connection” (Kim and Camilleri, 2000).

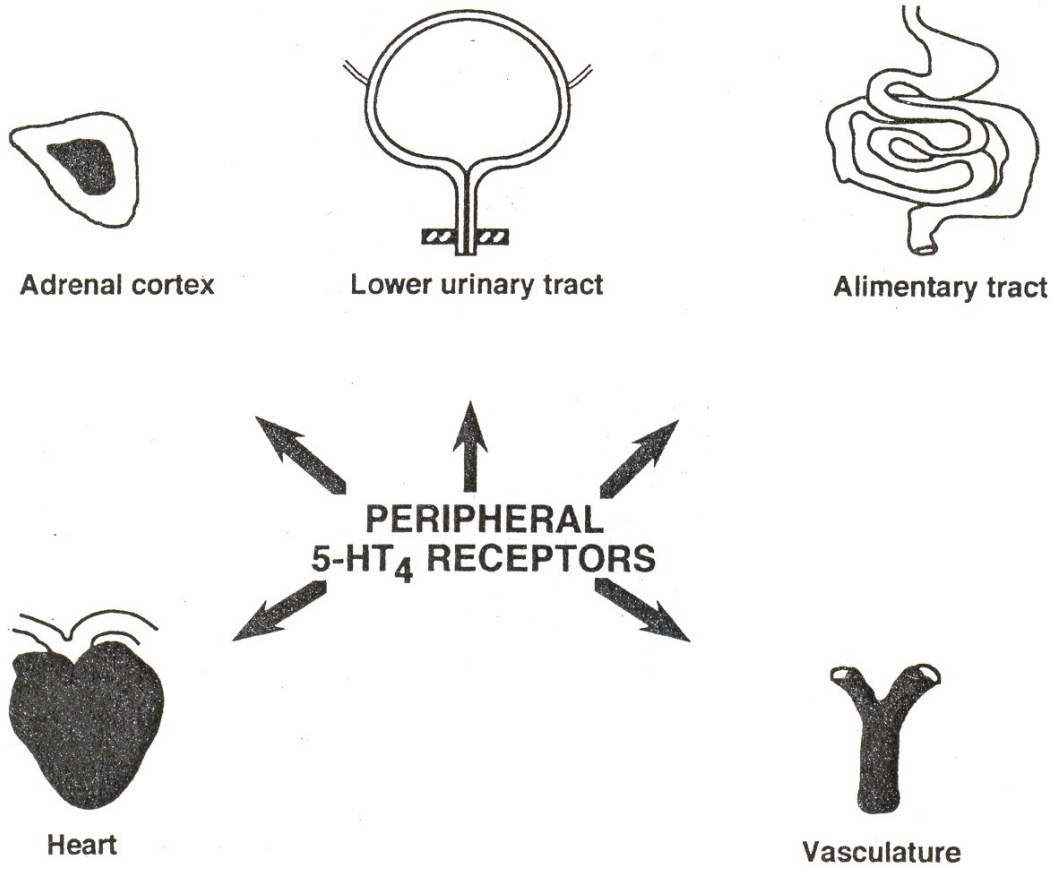


Fig. 1.15 *Location of 5-HT$_4$ receptors in various peripheral organs. Modified from Hegde and Eglen, 1996.*

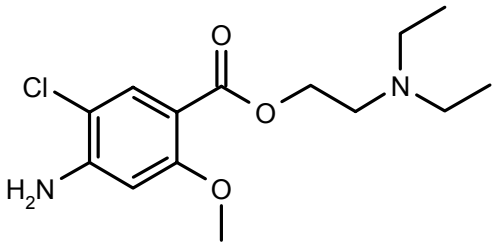
1.3.8 Ligands at 5-HT$_4$ receptors

MCP (Fig. 1.11) and tropisetron (Fig. 1.12) were used as starting points for the development of 5-HT$_4$ ligands. Prokinetic properties of MCP were published decades before the identification of the 5-HT$_4$ subtype (Jacoby and Brodie, 1967). Low affinity at this subtype was reported for tropisetron within the first characterisation of 5-HT$_4$ receptors (Dumuis et al., 1988). MCP had a low affinity and was neither a selective nor specific compound with comparable affinities to 5-HT$_4$, 5-HT$_3$ and D$_2$ receptors. The benzamide MCP and its analogues cisapride and zacopride (both Fig. 1.17) were reported to be full agonists or partial agonists at 5-HT$_4$ receptors with prokinetic actions (Buchheit and Buhl, 1991). With the ester congener SDZ205-557 (Fig. 1.16) of the benzamide MCP one of the first potent 5-HT$_4$ antagonists was identified (Buchheit et al., 1991, 1992), however, with only little subtype selectivity versus

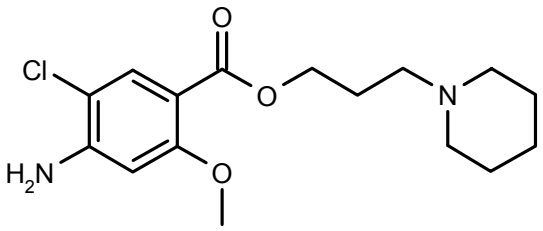
5-HT_3. Better selectivity was achieved with the piperidine analogue RS23597 (Eglen et al., 1993b). The first highly potent compound with excellent selectivity was reported with SB204070 (Fig. 1.16) with an affinity in the picomolar range (Gaster et al., 1993). The big problem of all previously reported compounds was their ester moiety, making them not useful as drugs due to rapid degradation. One of the first orally active compounds lacking the ester group was RS36904 (Fig. 1.16) with high affinity and selectivity (Hegde et al., 1995). Starting from tropisetron the highly potent and selective indole amide GR113808 (Fig. 1.16) was identified (Gale et al., 1994; Langlois et al., 1994). The tritiated form emerged to the most frequently used radioligand in the field of 5-HT_4 binding studies (Grossman et al., 1993). ML10302 (Fig. 1.17) was one of the first selective compounds displaying intrinsic activity at 5-HT_4 receptors (Elz and Keller, 1995). The benzimidazolone BIMU1 (Fig. 1.17) was shown to possess agonistic properties, too (Lelong et al., 2003). Recently, the serotonin analogue tegaserod (Fig. 1.17) was approved to the market as a partial agonist at 5-HT_4 receptors.

1.3.9 Functional models

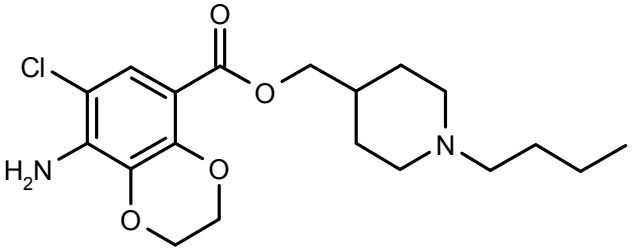
Several *in vitro* models for 5-HT_4 receptors were established. In guinea-pig ileum the ability of 5-HT agonists to enhance the contraction evoked by electrical field stimulation was measured. Antagonist affinity was determined as a function of the inhibition of 5-HT induced contraction (Buchheit et al., 1985; Tonini et al., 1992). The ability of 5-HT to relax rat oesophagus (ROS) tunica muscularis mucosae precontracted with carbachol was extensively used to determine 5-HT_4 affinity (Baxter et al., 1991). Interestingly, this model was not adaptable to other species such as guinea-pig, rabbit and dog (Cohen et al., 1994). The most commonly used *in vivo* models are gastrointestinal motility models in rat and mouse (Banner et al., 1993) in which 5-HT_4 agonists elicit prokinetic responses. In the anaesthetised mini-pig the effect of 5-HT_4 ligands on heart rate was examined. Agonists induced tachycardia, whereas antagonist affinity could be determined as the inhibition of 5-HT-induced tachycardia (Eglen et al., 1993a).



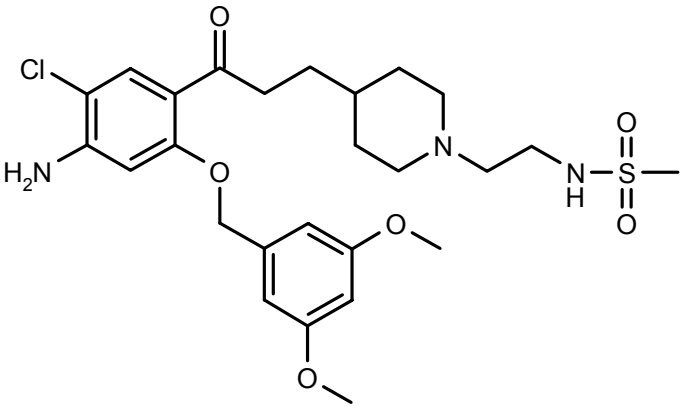
SDZ205-557



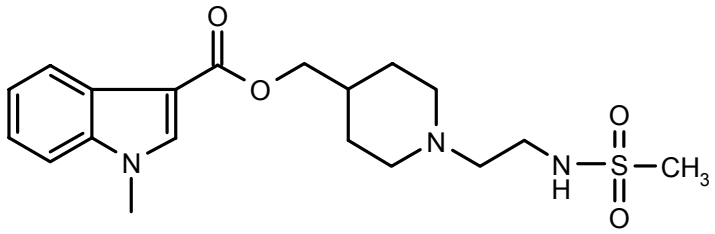
RS23597



SB204070



RS36904



GR113808

Fig. 1.16 *Chemical structures of selected 5-HT_4 antagonists.*

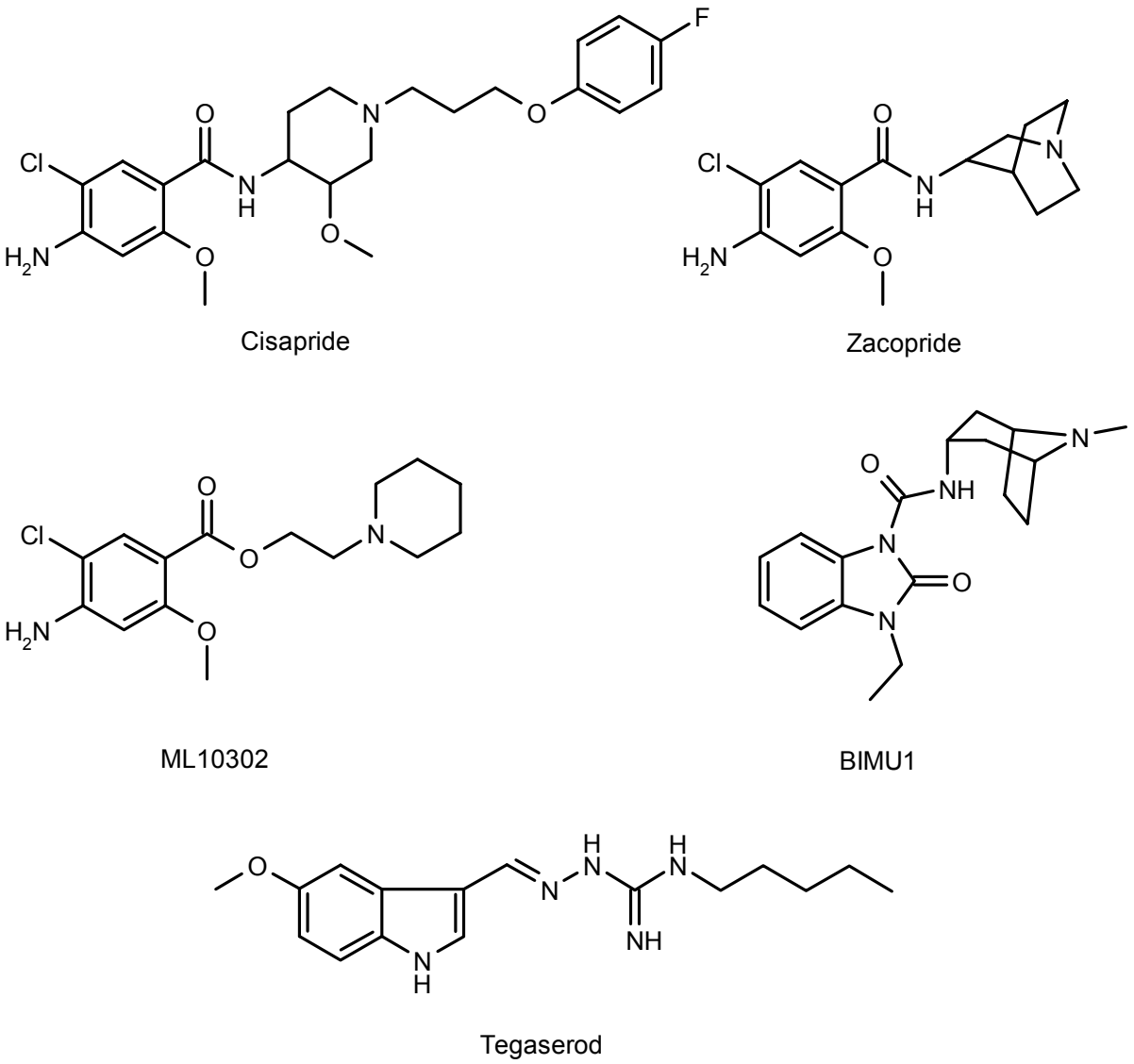


Fig. 1.17 *Chemical structures of selected 5-HT$_4$ agonists.*

1.3.10 Therapeutic options

1.3.10.1 Cardiac arrhythmia

5-HT induces rate-dependent arrhythmic contractions in human and pig heart *in vivo* via 5-HT$_4$ receptors located in the atria (not ventricles), that can be blocked with 5-HT$_4$ antagonists (Kaumann and Sanders, 1994). It was proposed that 5-HT$_4$ antagonists might become interesting new drugs to prevent atrial flutter and stroke (Kaumann, 1994) beside ß-blocker and Ca^{2+} channel blocker, without effects on the ventricles.

1.3.10.2 Urinary incontinence

5-HT_4 receptors were identified in the human bladder located on cholinergic fibres facilitating ACh release in human detrusor muscle (Tonini et al., 1994). As one could expect, activation of these receptors led to increased micturation frequency, reported as a side effect in patients treated with cisapride (Boyd and Rohan, 1994). It was proposed that 5-HT_4 agonists might be suitable new drugs to enhance cholinergic tone in urinary voiding disorders associated with detrusor failure due to low cholinergic tone (Tonini and Candura, 1996).

1.3.10.3 Cognition enhancement

With the discovery of a high amount of 5-HT_4 receptors in hippocampal structures an important role in memory and cognition was suggested (Waeber et al., 1994). Many reports were given describing the influence of 5-HT_4 receptors on the release of various neurotransmitters including ACh, 5-HT and DA in the brain (for review see Barnes and Sharp, 1999). Reports concerning 5-HT_4 receptors in anxiety disorders were conflicting. More hopeful results were reported in memory disorders. It was demonstrated that 5-HT_4 agonists ameliorated a scopolamine-induced memory impairment in rats (Matsumoto et al., 2001). Local application of a 5-HT_4 agonist into distinct brain regions resulted in increased release of ACh. Therefore, future research on highly selective and potent 5-HT_4 agonists and partial agonists is of great interest with regard to therapy of memory disorders such as AD.

1.3.10.4 Gastrointestinal disorders

Several receptors belonging to the serotonergic family were detected in the GIT influencing secretion, sensation and peristalsis. Intestinal secretion is enhanced directly via 5-HT_4 receptors located on enterocytes and indirectly via 5-HT_3 receptors on secretory mucosal nerves and vagal afferents (Spiller, 2002). Visceral sensation is mediated through 5-HT_3 receptors located on vagal afferents. A key role for control of peristalsis accounts to 5-HT release out of enterochromaffin cells transducing a luminal pressure signal in a nervous stimulus. Fig. 1.18 summarises the network involved in the peristaltic reflex and the role of 5-HT_4 receptors. An ascending stimulus of circular muscle contraction together with a descending relaxation of

circular muscles results in a caudal propulsion. Prokinetic properties in human and animals were described for MCP long before the identification of 5-HT_4 receptors.

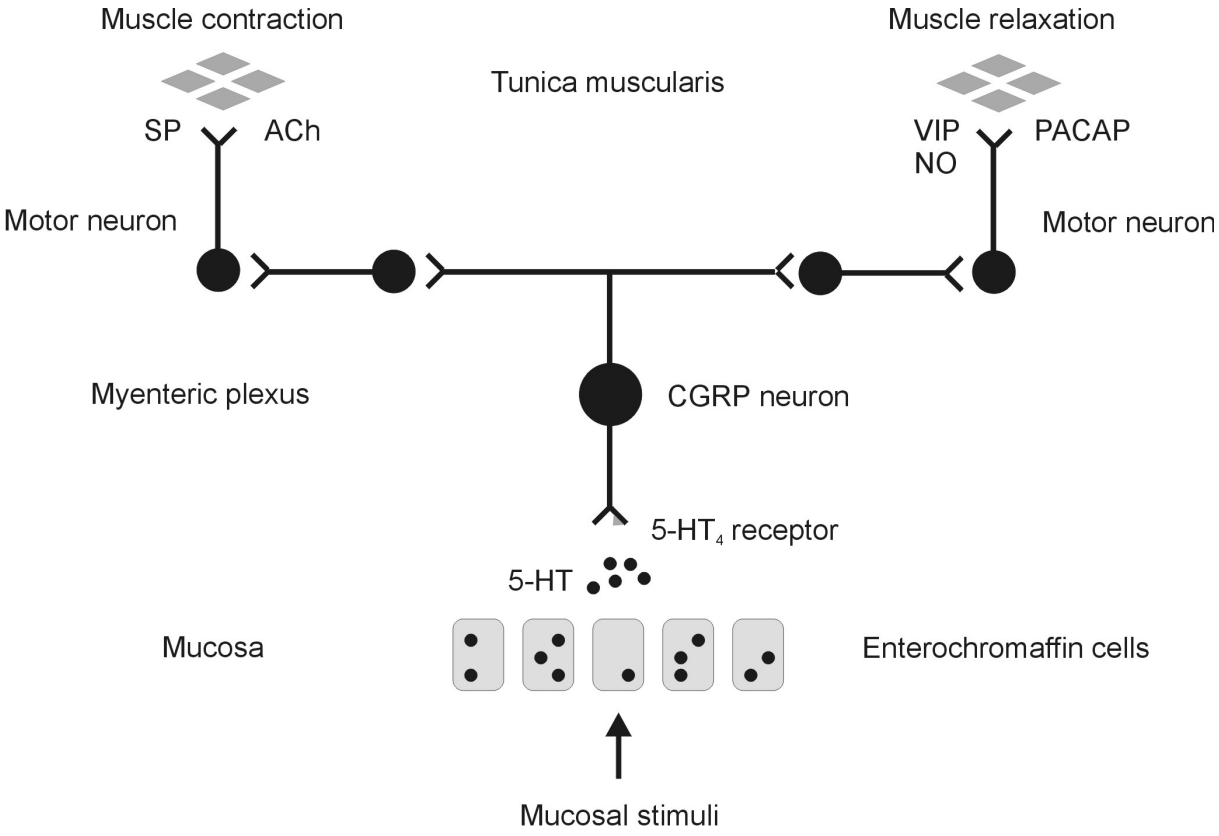


Fig. 1.18 *Model of 5-HT effect on activation of intrinsic afferent neurons in the lamina propria after being released from enterochromaffin cells. Activation of CGRP neurons (**c**alcitonin **g**ene **r**elated **p**eptide) stimulates myenteric neurons to activate the "peristaltic reflex". This involves an oral contraction mediated through ACh and substance P and an caudal relaxation mediated through NO, VIP (**v**asoactive **i**ntestinal **p**eptide) and PACAP (**p**ituitary **a**denylate **c**yclase-**a**ssociated **p**eptide). Adapted from Camilleri, 2001 and Langlois and Fischmeister, 2003.*

In the following a short focus is laid on the irritable bowel syndrom (IBS). IBS is the most common non-infectious diagnosis in GIT and secondly only to the common cold as a cause of work absenteeism. With 10 - 20 % of all individuals being affected from IBS, resulting in enormous costs to public health care, an enormous burst in research took place that was extensively reviewed (Thompson, 2002; Camilleri et al., 2002). IBS is defined as a group of functional bowel disorders in which abdominal discomfort or pain is associated with a change in bowel habit and with features of

disordered defecation. Diagnosis is made by exclusion of other organic diseases. Three subtypes of IBS are differentiated based on the predominant bowel habit: **c**onstipation, **d**iarrhoea and **a**lternating bowel movements abbreviated C-IBS, D-IBS and A-IBS, respectively. Currently available drug therapy is far from satisfying. Anticholinergics, low-dose antidepressants, dietary fibre and opioid agents are used with little efficacy next to more experimental approaches and psycho-therapy. With new insights in the role of the serotonergic systems in the GIT, 5-HT_3 and 5-HT_4 receptors were identified as new target sites for IBS treatment.

In summary, 5-HT_4 agonists may potently increase motility and therefore might represent useful drugs in C-IBS, functional constipation, gastro-oesophageal reflux disease and dyspepsia (Spiller, 2002; Hansen, 2003). The lately approved 5-HT_4 partial agonist tegaserod showed increased gastric emptying, accelerated small and large bowel transit as a potent prokinetic agent in women, reduced pain associated with defecation and ameliorated hypersensitivity to rectal distension (Lacy and Yu, 2002; Zimmermann, 2002). 5-HT_3 antagonists are of interest for D-IBS as alosetron decreased abdominal pain, colon hypersensitivity and motility in D-IBS women. For some GIT disorders, such as dyspepsia and gastro-oesophageal reflux disease, it is still not clear whether 5-HT_3 antagonists, 5-HT_4 agonists, a combination or non of both is suitable for therapy.

1.4 Radioligand binding studies

Radioligand binding studies have become a more and more important tool within the drug discovery process as a robust and fast screening method, rapidly providing binding characteristics of compounds at several target sites (Williams, 1991). Identification and characterisation of receptor sites in tissue preparations is another suitable application for this method. Radioligand binding studies provide valid data when experimental conditions are chosen carefully. Incubation temperature, pH value, ionic strength and composition of incubation buffer are critical parameters within the testing procedure and strongly influence the result (Asselin et al., 1983; Pedder et al., 1991; Hou et al., 1996). Attention should also be paid to incubation time because it is important to reach an equilibrium state within the tests to assure

correct data analysis. However, binding studies merely provide an affinity estimate for a compound at a specific target site and give no direct information about potential intrinsic activity. Agonists often show shallow, sometimes biphasically shaped competition curves resulting from the existance of multiple agonist affinity states (Birdsall et al., 1978). The assessment of agonist affinity and efficacy in binding studies is difficult. There are some approaches to determine these properties that allow a comparison of agonist potencies in a semi-quanitative way (for review see Christopoulos and El Fakahany, 1999), but results may not totally reflect reality. In summary, binding studies provide a fast and robust method to determine affinity values for antagonists but are not favourable to determine agonist properties.

1.5 Recombinant receptor systems

In native tissues there is often a diversity of receptors expressed that belong to one neurotransmitter system. This makes it difficult to address affinity to a single receptor subtype in radioligand binding studies unless highly selective radioligands are available. This problem could be eliminated with the recombinant expression of a single receptor in suitable cell lines. Recombinant cell lines provide easy access to a constant, high receptor population without using laboratory animals. However, different expression systems provide a different cellular machinery for posttranslational modifications, resulting in differences to the receptors expressed in native tissues. Additionally, the receptor expression in recombinant systems is often very different from physiological density. An advantage of recombinant systems is that human receptors can be used instead of receptors of laboratory animals. This is important as huge species differences were reported for some receptor subtypes between humans and laboratory animals.

1.6 Stereochemistry

Enantiomers are composed of the same number and kinds of atoms bonded in an identical fashion, but differ in their three-dimensional orientation. Enantiomers share the same physical and chemical properties and behave identical in an achiral environment. Binding sites at receptors represent a chiral environment and form

diastereomeric complexes with chiral ligand molecules, most probably resulting in different binding properties for enantiomers. The difference in pharmacological activities of the stereoisomers of chiral compounds is called “stereoselectivity” (Lambrecht and Mutschler, 1986). Special terms were introduced to distinguish enantiomers with regard to their pharmacological properties at a special target site. The enantiomer with higher affinity is called “eutomer”, the one with lower affinity “distomer” (Lehmann, 1986).

2 Aims

2.1 General considerations

In this work structure-activity relationship (SAR) research in the field of several receptor families was carried out, including the serotonergic, histaminergic and muscarinic systems. Each receptor family is associated with different therapeutic implications and problems in drug development. Thus, it is of general interest to obtain more information about the relevance of molecular modifications at a chosen lead structure concerning affinity, subtype selectivity and specificity to design new drug candidates providing an appropriate binding profile. Improved binding characteristics may open new therapeutic options, improve patient compliance and drug efficacy or decrease side effects. Modification of kinetic properties may result in a prolonged time of action or kinetic selectivity. One important feature of this work was to highlight the influence of stereochemistry on absolute binding affinities as well as on kinetic binding properties.

Compounds listed with "**(±)**" were tested as racemic mixtures, compounds with "**(+/-)**" or "**(R/S)**" as pure enantiomers. In some series "**(±)**" was omitted to keep nomenclature less complicated.

2.2 Analogues of ondansetron

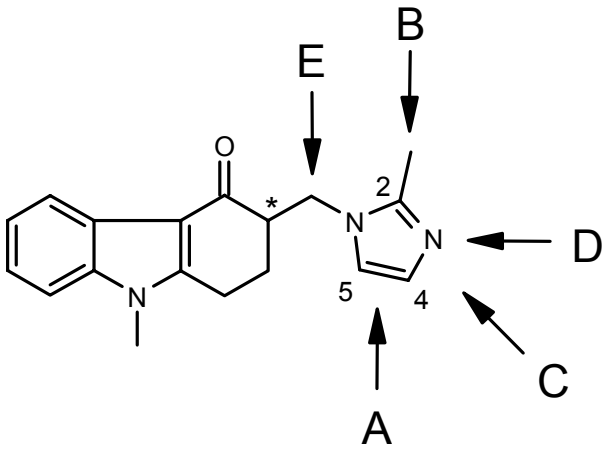
The 1-imidazolylmethyl substituted tetrahydrocarbazolone ondansetron (**1**) can be considered as a prototypic 5-HT_3 antagonist displaying high affinity to this subtype. It is widely used in therapy to prevent nausea and vomiting induced by chemo- and radiotherapy. Ondansetron was characterised already in 1987 (Kilpatrick et al., 1987) in binding studies using a rat brain tissue preparation confirming its high affinity to native 5-HT_3 receptors. Since then, extensive research efforts were undertaken to find derivatives with even higher affinity and increased subtype selectivity. SAR work led to the discovery of alosetron (Fig. 1.12), a closely related molecule of **1**, possessing the highest affinity of all approved 5-HT_3 antagonists. As patents for older drugs are running out, there is still a major demand for new therapeutic options (e.g. in IBS). Therefore, new compounds displaying high 5-HT_3 affinity are still of interest.

In our study a series of 23 analogues (compounds **2A/B** - **6A/B** and **7** - **18**) of ondansetron were evaluated for their binding affinities at cloned h5-HT_{3A} receptors

stably expressed in HEK293 cells. Several of these molecules were previously shown to possess up to subnanomolar affinities at 5-HT_{2A} receptors in functional studies using a rat tail artery model (Elz and Heil, 1995). All compounds were already tested at functional 5-HT_3 receptors in guinea-pig ileum by in the group of Prof. Dr. S. Elz, Regensburg, Germany, displaying affinities up to pA_2 = 7.4. We wanted to confirm these data and performed a comparative study at functional, native gp5-HT_3 and recombinantly expressed h5-HT_{3A} receptors. Ondansetron (**1**) as starting point served as a reference drug.

In Table 2.1 the formula of ondansetron is given with arrows indicating the general sites of modifications. All modifications left the tetrahydrocarbazolone structure unchanged and took place in the side chain and / or imidazole ring.

Table 2.1 *Structure of ondansetron (**1**) and general sites of structural variations. The asterisk denotes the centre of chirality.*



A Introduction of substituents at position 4 / 5

B Modifications of substituent at position 2

C Condensing an aromatic ring or exchange of imidazole → piperidine

D Quaternization of nitrogen

E Changes in side chain and / or imidazole moiety

Compounds with the following modifications were tested in this study:

- Compounds with changed substitution pattern in the imidazole structure. The proton in position 4 or 5 of the imidazole structure was replaced by substituents of increasing size - ranging from methyl (**2A/B**) up to a ethylphthalimide moiety (**6A/B**) - to find out which site is preferred for substitution. Compounds carrying substituents in position 4 are termed with "**A**", those with substituents in position 5 with "**B**". Table 2.2 gives the formula of compounds **2A/B** - **6A/B**.

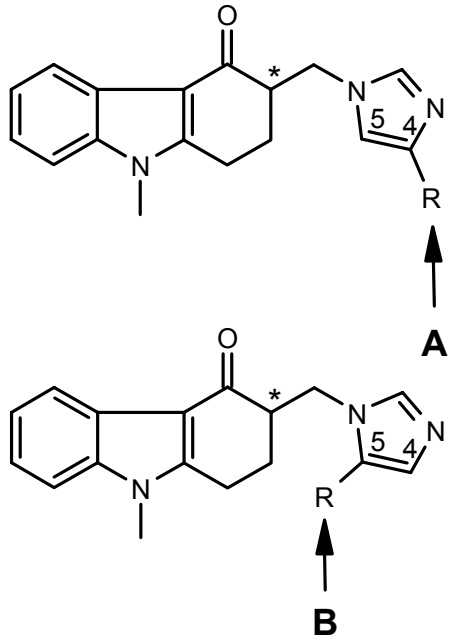
- Modifications of the substituent in position 2 of the imidazole structure. Introduction of a basic nitrogen with modification in alkylation pattern were tested in compounds **7** - **10** shown in Table 2.3.

- N-Methylation of the nitrogen atom in position 3 of the imidazole moiety lead to the quaternary molecule **11** and enlargement of the basic imidazole structure by condensing of a phenyl ring to compound **12**. Exchange of the imidazole structure by a piperidine ring led to compound **13**, and the p-substituted compounds **(+)-14** and **(-)-14**, which were tested as pure enantiomers. **15** displayed a sterically fixed, bulky substituent attached in position 4 of the piperidine ring. Structures of compounds **11** - **17** are summarised in Table 2.4.

- Changes in the connecting side chain and / or the imidazolyl structure led to compounds **16** - **18** (Table 2.5).

All compounds with exception of **(+)-14** and **(-)-14** were tested as racemates.

Table 2.2 *Compounds with substituents in position 4 or 5 of the imidazole structure. Compounds were monosubstituted each with a hydrogen at the non-substituted atom. The asterisks denote the centres of chirality.*



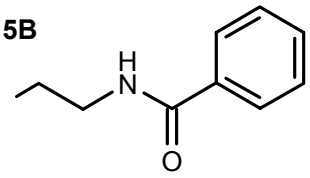
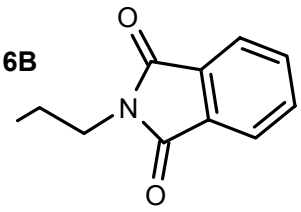
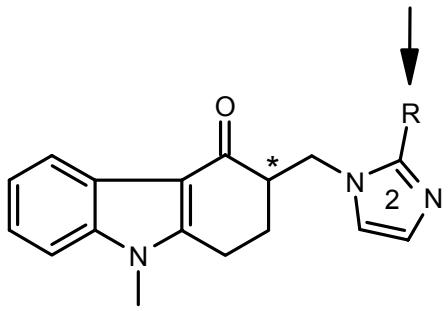
No.	R	No.	R
2A / 2B	CH_3	**5A / 5B**	
3A / 3B	CH_2OH	**6A / 6B**	
4A / 4B	$C_2H_4NH_2$		

Table 2.3 *Analogues with modifiction in position 2 of the imidazole moiety. The asterisk denotes the centre of chirality.*



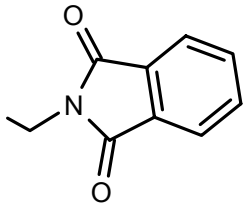
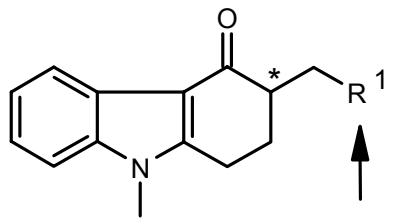
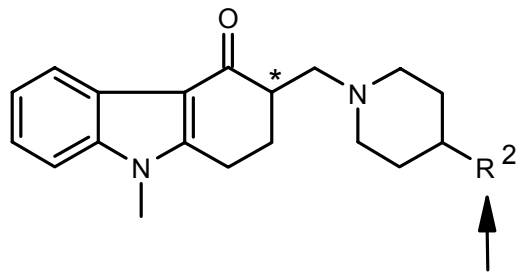
No.	R	No.	R
7	$NHCH_3$	**9**	CH_2NH_2
8	$N(CH_3)_2$	**10**	

Table 2.4 *Derivatives with a quarternary nitrogen, condensed aromatic ring or piperidine ring. The asterisks denote the centres of chirality.*





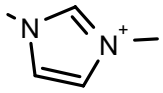
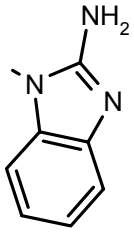
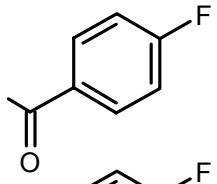
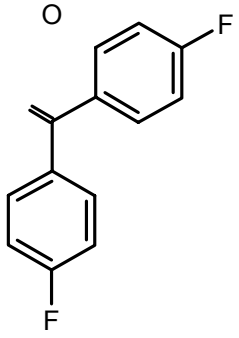
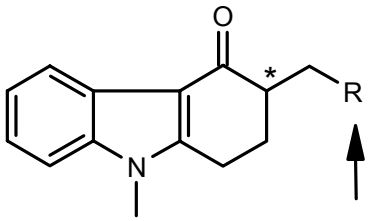
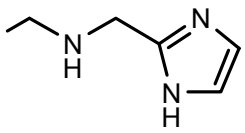
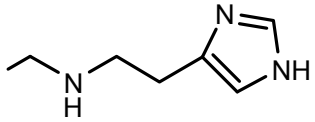
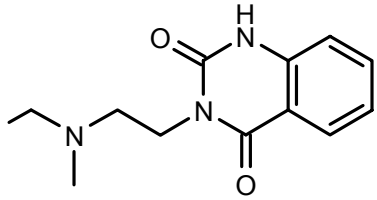
No.	R^1	No.	R^2
11		13	H
12		(+/-)-14	
		15	

Table 2.5 *Analogues with modifications in the side chain and imidazole structure. The asterisk denotes the centre of chirality.*



No.	R
16	
17	
18	

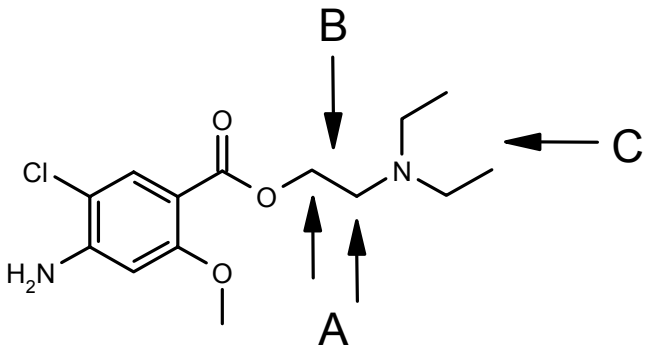
2.3 Analogues of metoclopramide

Metoclopramide (MCP), which can be considered as the starting point in research for compounds with prokinetic activity, displayed only low affinity to 5-HT_4 receptors in several test models and insufficient subtype selectivity. A vast amount of research was done in the last 40 years to obtain new compounds related to MCP with improved characteristics. One step forward was made by exchanging the amide group of MCP into an ester group (Buchheit et al., 1991) leading to SDZ205-557 (**19**), showing increased affinity and improved subtype selectivity at 5-HT_4 receptors in functional tests at guinea-pig ileum. Currently, several 5-HT_4 receptor ligands are under investigation in clinical trials as new drug candidates for different indications. Most drugs in clinical trials displayed partial agonism at the 5-HT_4 subtype. Even though many partial agonists were under investigation, only tegaserod as a newer compound with affinity to 5-HT_4 receptors was approved in some countries for use in gastrointestinal disorders. In contrast, up to now, no 5-HT_4 antagonist has reached the market. Since the withdrawal of cisapride due to cardiac side effects only MCP and domperidone are left on the market as synthetic prokinetic drugs.

In this study we examined the binding affinities of SDZ205-557 (**19**) and its analogues **20** - **30** at cloned h5-$HT_{4(b)}$ receptors in a SAR study. All compounds were previously tested in functional studies at 5-HT_4 receptors in guinea-pig ileum and some in rat oesophagus in the group of Prof. Dr. S. Elz, Regensburg, Germany. The values obtained at native gp5-HT_4 and r5-HT_4 receptors in functional studies were compared with binding affinities obtained in experiments with recombinantly expressed h5-$HT_{4(b)}$ receptors.

Table 2.6 shows the formula of SDZ205-557 (**19**) which served as a starting point in this SAR study. Arrows indicate the general sites of modifications. All compounds had an unchanged 4-amino-5-chloro-2-methoxybenzoic acid structure and were ester analogues of MCP with different amino alcohols.

Table 2.6 *Structure of SDZ205-557 (**19**) and general sites of structural variations.*



A	Methylation of the side chain creating centres of chirality
B	Modification of side chain length
C	Changing the structure of the amino group → piperidine or piperazine

The following molecule variations were tested in our study:

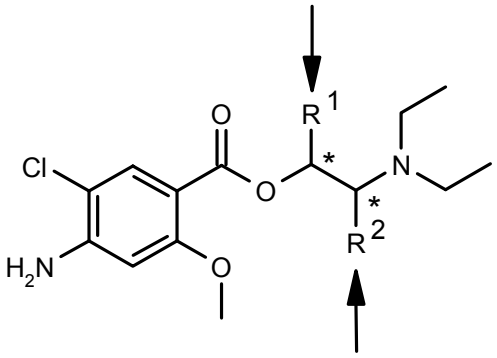
- Compounds with methylation in the side chain, **(±)-20** and **(±)-21**. These compounds possess a centre of chirality in contrast to the unsubstituted mother compound **19**. The structures are given in Table 2.7.

- Insertion of the basic, tertiary nitrogen in a piperidine ring system and modification in the side chain delivered compounds **22** - **26** shown in Table 2.8.

- Insertion of the basic, tertiary nitrogen in a piperazine ring system, modification of side chain length and different substituents at the p-nitrogen of the piperazine ring lead to structures **27** - **30** displayed in Table 2.9.

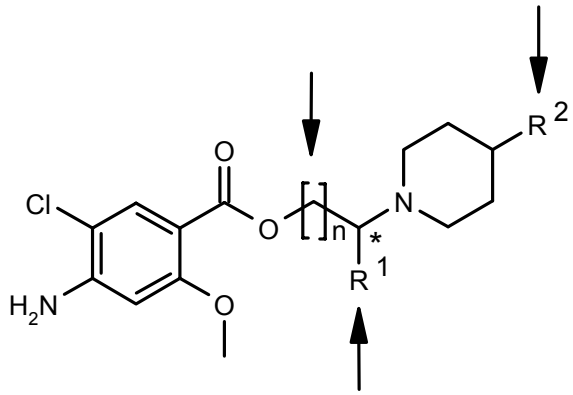
Chiral compounds **(±)-20**, **(±)-21** and **(±)-26** were tested as racemates, **(+)-23** and **(-)-23** as pure enantiomers.

Table 2.7 *Compounds with a methyl group in the side chain. The asterisks denote the centres of chirality.*



No.	R^1	R^2
19	H	H
(±)-20	CH_3	H
(±)-21	H	CH_3

Table 2.8 *Compounds with a piperidine ring system, modifications in the side chain length and methylation pattern. The asterisk denotes the centre of chirality.*



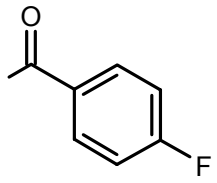
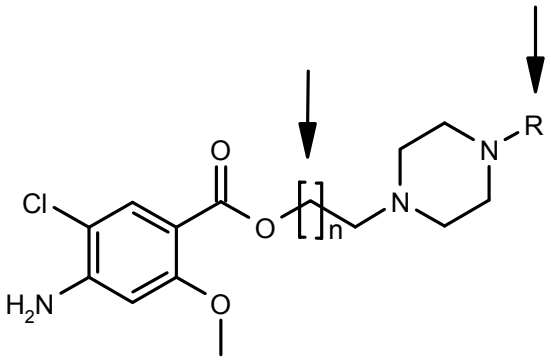
No.	n	R^1	R^2
22	**1**	H	H
(+/-)-23	**1**	CH_3	H
24	**2**	H	H
25	**3**	H	H
(±)-26	**1**	H	

Table 2.9 *Compounds with a piperazine ring and modifications of side chain length.*



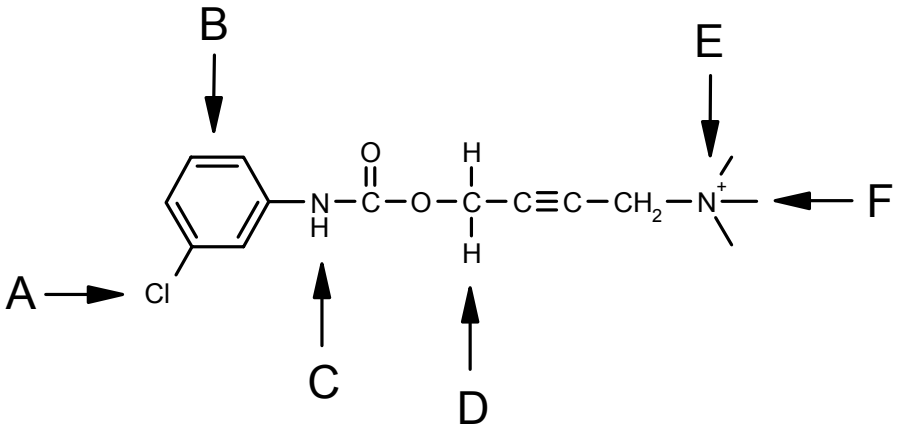
No.	n	R
27	**1**	Benzyl
28	**2**	Benzyl
29	**3**	Benzyl
30	**1**	Phenyl

2.4 Analogues of McN-A-343

McN-A-343 (**31**) has a long history as a compound showing affinity to muscarinic ACh receptors since the 1960s of the last century (Roszkowski, 1961). Several publications on SAR concerning functional and binding characteristics at muscarinic receptors were published (Nilsson et al., 1992; Lambrecht et al., 1993, 1995). However, comparably little is known about binding affinities of **31** and its derivatives at other target sites such as histamine H_1 and serotonin 5-HT_3 and 5-HT_4 receptors. **31** was reported to display affinities to 5-HT_3 and 5-HT_4 receptors in binding studies as an atypical chemical structure in this pharmacological class (Sagrada et al., 1994). We wanted to confirm these results and provide new SAR information at these subtypes for some related compounds. It is well known that muscarinic ACh receptors are closely related to histamine H_1 receptors. In our laboratory, former tests revealed affinity values at $M_{1\text{-}5}$ receptors spanning 5 orders of magnitude for derivatives of **31** up to picomolar concentrations. We investigated binding affinities at histamine H_1 receptors with a special focus on stereochemistry. McN-A-343 (**31**) and its derivatives **32** - **42** were examined in binding studies at hH_1, h5-HT_{3A} and h5-$HT_{4(b)}$ receptors.

Table 2.10 shows the formula of McN-A-343 (**31**) which served as a starting point in this SAR study. Arrows indicate the general sites of modifications. All compounds were tested as tertiary amines and their corresponding N-methylated compounds generally marked with "$^{+}$".

Table 2.10 *Structure of McN-A-343 (**31**) and general sites of structural variations.*



A	Changes in substitution pattern
B	Exchange of the aromatic ring
C	Removal of NH: carbamate → carboxylic acid ester
D	Exchange of H atom → chiral compounds
E	N-Methylation → quaternary compounds
F	Changing the amino group → pyrrolidine ring

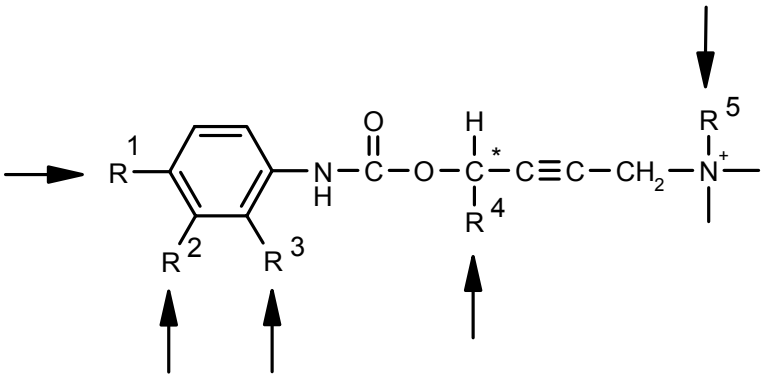
The following molecule variations were tested in our studies:

- Compounds with an acyclic amino group and 4-F-substitution (**32**). Chiral derivatives with a phenyl ring attached to the carbon atom next to the carbamate structure in the chain, **(R/S)-33**, **(±)-34**, were tested. Structures are given in Table 2.11.

- Compounds containing a pyrrolidine ring system with increasing size of substituents in the chain (Table 2.12), ranging from a simple methyl group in **(R/S)-35** to a bulky naphthyl substituent in **(±)-39**.

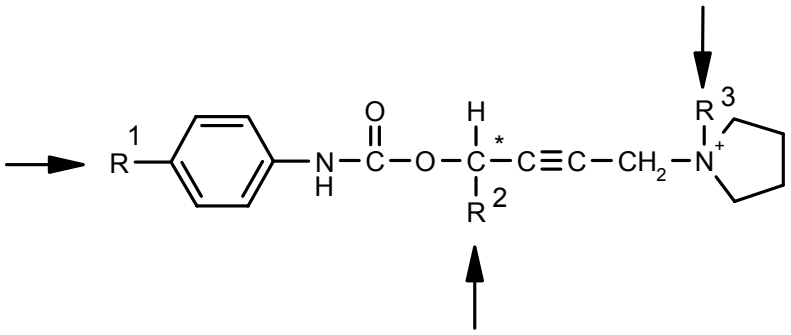
- Compounds with exchanged aromatic moieties, **(±)-40** and **(±)-41**, and an ester congener, **(±)-42**, with replaced carbamate group (Table 2.13).

Table 2.11 *Derivatives with acyclic amino group and changes in substituents at the aromatic ring and chain. The asterisk denotes the centre of chirality.*



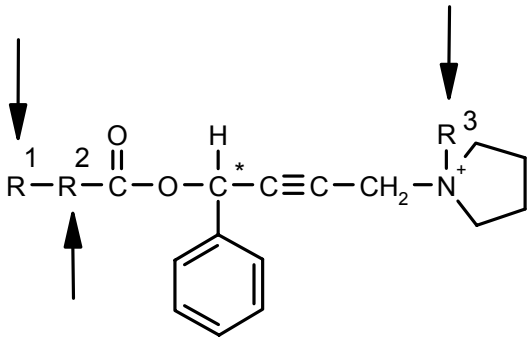
No.	R^1	R^2	R^3	R^4	R^5
31	H	Cl	H	H	CH_3
32	F	H	H	H	H
32⁺	F	H	H	H	CH_3
(R/S)-33	H	H	H	Phenyl	H
(R/S)-33⁺	H	H	H	Phenyl	CH_3
(±)-34	H	H	Phenyl	Phenyl	H
(±)-34⁺	H	H	Phenyl	Phenyl	CH_3

Table 2.12 *Derivatives with a pyrrolidine structure and changes in substituents at the aromatic ring and chain. The asterisk denotes the centre of chirality.*



No.	R^1	R^2	R^3
(R/S)-35	F	CH_3	H
(R/S)-35⁺	F	CH_3	CH_3
(R/S)-36	F	Phenyl	H
(R/S)-36⁺	F	Phenyl	CH_3
(R/S)-37	H	Phenyl	H
(R/S)-37⁺	H	Phenyl	CH_3
(±)-38	F	4-F-Phenyl	H
(±)-38⁺	F	4-F-Phenyl	CH_3
(±)-39	F	1-Naphthyl	H
(±)-39⁺	F	1-Naphthyl	CH_3

Table 2.13 *Compounds with a pyrrolidine ring system, exchanged aromatic moieties and variation in the carbamate structure. The asterisk denotes the centre of chirality.*



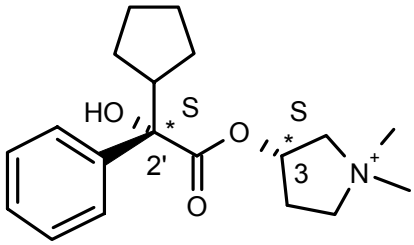
No.	R^1	R^2	R^3
(±)-40	1-Naphthyl	NH	H
(±)-40⁺	1-Naphthyl	NH	CH_3
(±)-41	$(Phenyl)_2$-CH	NH	H
(±)-41⁺	$(Phenyl)_2$-CH	NH	CH_3
(±)-42	4-F-Phenyl	-	H
(±)-42⁺	4-F-Phenyl	-	CH_3

2.5 Analogues of glycopyrronium

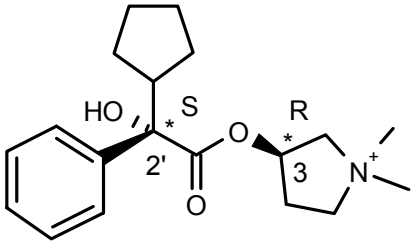
Since many years glycopyrronium (**43**) is used in therapy as premedication prior to surgical operations as a parasympatholytic drug and rarely as a spasmolytic drug, too (Mirakhur and Dundee, 1983). Some investigations were made in topical use of **43** to reduce excessive sweating (Atkin and Brown, 1996). Glycopyrronium is a quaternary compound sold as a bromide salt. The glycopyrronium molecule displays two centres of chirality resulting in four stereoisomers (Table 2.14). Actually, a mixture of the enantiomers (3S, 2'R)- and (3R, 2'S)-glycopyrronium, named glycopyrrolate, is approved and in therapeutic use. The 2'-centre is located in the acid part of the molecule, the 3-centre in the basic amino-alcohol. In former tests in our laboratories SAR research in the field of glycopyrronium and its derivatives was carried out by K. Kreutzmann. He showed the (2'R)-configurated isomers of **43** to be more potent than the (2'S)-configurated congeners and only little influence on binding affinity at muscarinic subtypes of the absolute configuration at the second centre of chirality (personal communication). All tested compounds were non-selective at muscarinic receptor subtypes. Even more interesting was his finding that the (3R, 2'R)-configurated isomer of **43** displayed a long dissociation half-life at the M_3 subtype compared to the other subtypes, resulting in kinetic selectivity. This characteristic was also described for the drug tiotropium, recently approved for COPD, resulting in a superior once-daily dosing regimen and prolonged action of this new drug compared to older drugs currently in use e.g. ipratropium and oxitropium (Barnes, 2001). Compounds corresponding to **43** with two centres of chirality have the letters **A-D** attached to the compound number, describing the absolute configuration of derivatives as follows: **A**: (3S, 2'S); **B**: (3R, 2'S); **C**: (3S, 2'R); **D**: (3R, 2'R). Table 2.14 gives the structures of the four stereoisomers of **43**. The letters "**R**" and "**S**" in the tables standing next to an asterisk describe the absolute configuration of that centre of chirality.

Table 2.15 gives the structure of glycopyrronium (**43**) which served as a starting point in this SAR. Arrows indicate the general sites of modifications.

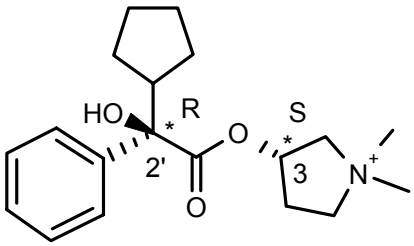
Table 2.14 *Structures of the four possible stereoisomers of **43**.*



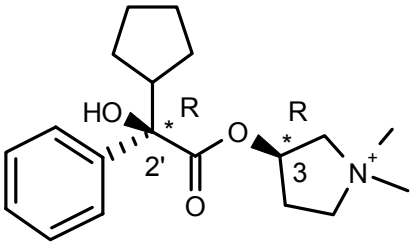
A: 3S, 2'S



B: 3R, 2'S

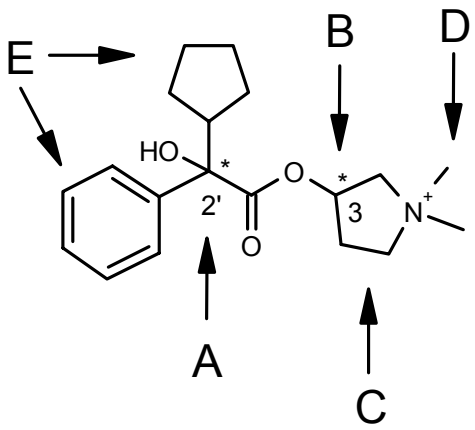


C: 3S, 2'R



D: 3R, 2'R

Table 2.15 *Structure of glycopyrronium and general sites of structural variations. The asterisks denote the centres of chirality.*



A	Changing the absolute configuration of 2'-centre of chirality
B	Changing the absolute configuration of 3-centre of chirality
C	Exchange of amino-alcohol: pyrrolidine → chinuclidine
D	N-Alkylation → chiral compounds with an asymmetric nitrogen
E	Exchanging the aromatic and/or aliphatic rings

All tested compounds were quaternary molecules containing an ester group. The following molecule variations were tested in our studies:

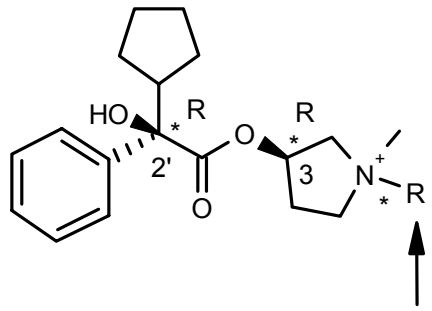
- Compounds with a pyrrolidine structure in the alcoholic part. Analogues **Dia-44D** - **Dia-48D** (Table 2.16) had a stereochemical configuration identical to **43D** (3R, 2'R) with modifications in N-alkylation. These compounds are mixtures of cis-trans isomers at the nitrogen atom. It is important to note that these compounds possess one more centre of chirality, namely the quaternary nitrogen atom. This makes a total of 8 possible stereoisomers. Compounds **Dia-44D** - **Dia-48D** were tested as racemic mixtures at this new stereocentre.

- Compounds with a chinuclidine structure as an amino alcohol. All stereoisomers of the methyl-substituted compounds **49A-D** were tested. **50D** and **51D** had modifications in N-alkylation. Table 2.17 shows the corresponding structures.

- Two molecules of **43D** were attached together to a dimer (**52D**) with an alkylene spacer. This molecule was tested as a mixture of four stereoisomers resulting from the chiral nitrogen atoms. Two synthesis precursors, **Dia-53** and **(R)-54**, were tested for affinity, in order to find out whether a smaller acid part of the molecule was sufficient for high affinities at muscarinic subtypes. **Dia-53** is a mixture of the two (R, R)- and (R, S)-diastereomers. Structures are shown in Table 2.18.

- Structural hybrids of tiotropium and glycopyrronium were tested. Compound **(R)-55** consisted of the acid part of glycopyrronium and the alcohol part of tiotropium. Derivative **(R)-56** possessed the acid part of tiotropium and a strucural variation to the amino alcohol of glycopyrronium. Both molecules were tested as pure (R)-configurated enantiomers. Structures are shown in Table 2.19. The structure of tiotropium is presented for reason of comparison.

Table 2.16 *Compounds with pyrrolidine ring and modifications in N-alkylation. The asterisks denote the centres of chirality.*



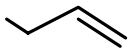
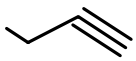
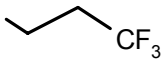
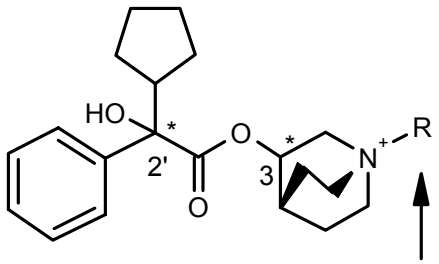
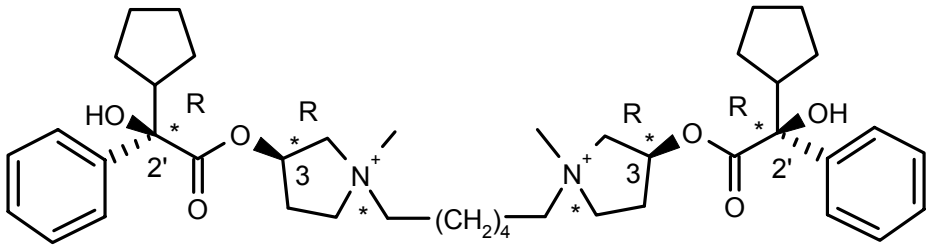
No.	R	No.	R
Dia-44D		**Dia-47D**	$(CH_2)_2$Phenyl
Dia-45D		**Dia-48D**	$(CH_2)_3$Phenyl
Dia-46D			

Table 2.17 *Compounds with chinuclidine ring and modifications in N-alkylation. The asterisks denote the centres of chirality.*

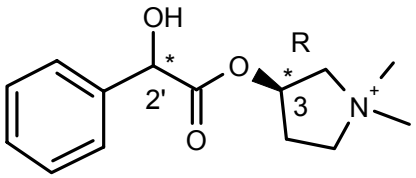


No.	R
49A-D	CH_3
50D	$(CH_2)_2$Phenyl
51D	$(CH_2)_3$Phenyl

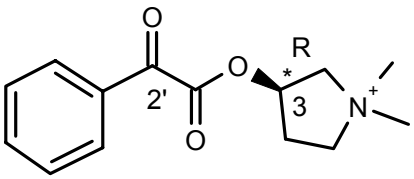
Table 2.18 *Dimerised structures and synthesis precursors. The asterisks denote the centres of chirality.*



No. 52

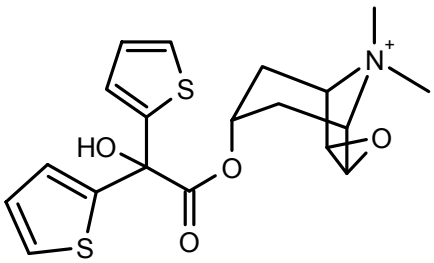


No. Dia-53

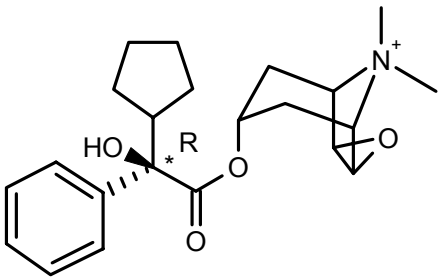


No. (R)-54

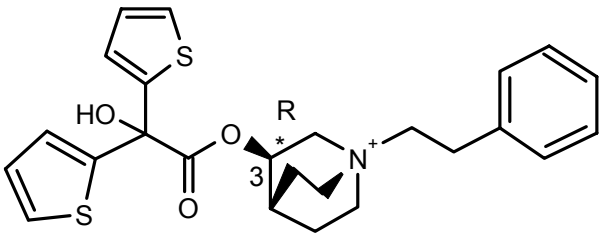
Table 2.19 *Hybrid molecules consisting of parts of tiotropium and glycopyrronium. The asterisks denote the centres of chirality.*



Tiotropium



No. (R)-55



No. (R)-56

2.6 Characterisation of [^{3}H](3R, 2'R)-glycopyrronium

Kinetic studies concerning the stereoisomers of glycopyrronium **43A-D** were carried out in our laboratory by K. Kreutzmann. He investigated association- and dissociation rate constants (k_{on} and k_{off}) of **43A-D** and of several analogues in experiments with cold, unlabelled compounds at muscarinic M_2 and M_3 receptors, using [^{3}H]NNMS as radioligand. He was able to show that dissociation half-lifes were very different for **43A-D** depending on the absolute configuration at the centres of chirality. Compounds with (2'R)-configuration showed much slower dissociation at the M_3 subtype compared to their (2'S)-configurated isomers. The longest $t_{1/2}$ at M_3 was found for the (3R, 2'R)-configurated stereoisomer **43D**. Further on, dissociation at the M_3 subtype was much slower compared to the M_2 subtype for **43D** ($t_{1/2}$ = 134 and 18 minutes, respectively, K. Kreutzmann, personal communication). This difference in dissociation half lives may lead to a kinetic selectivity as it was published for tiotropium (Disse et al., 1993). As it turned out to be difficult to find highly selective compounds at M_{1-5} receptors based on different affinities, the approach of kinetic selectivity is of particular interest. Tiotropium has already shown its superiority to older drugs on the market (ipratropium and oxitropium) concerning dosing regimen and efficacy in COPD therapy (Barnes, 2001). Structures of the quaternary compounds most commonly used in COPD therapy are given in Table 2.21. M_{1-3} receptors were found in human lung tissue. The M_3 subtype was found to be most important for direct bronchoconstriction in human airway smooth muscles (Bymaster et al., 2003). Therefore, antagonists with prolonged effect at M_3 and possessing little influence on the other subtypes expressed in human airways, are of great interest.

Cold-kinetic experiments carried out by K. Kreutzmann were an indirect way to obtain the kinetic constants of an unlabeled compound. For direct measurement of kinetic constants a labelled compound was necessary. Therefore, a cold precursor of the most interesting compound **43D** (synthesised in the group of Prof. Dr. C. Noe, Vienna, Austria) was sent to Amersham (Buckinghamshire, UK) for radioactive labelling. Table 2.20 shows the structure of labelled **[N-methyl-^{3}H](3R, 2'R)-glycopyrronium** used as trifluoroacetate, shortly **[^{3}H]43D**. In our studies we wanted to perform a complete characterisation of this compound. This implicated saturation-, competition-, and kinetic experiments. We wanted to confirm the kinetic constants

obtained in cold-kinetic experiments with the unlabelled drug and further on to determine the kinetic constants at all muscarinic receptor subtypes.

Table 2.20 *Structure of the labelled compound [^{3}H](3R, 2'R)-glycopyrronium ([^{3}H]43D) used as trifluoroacetate. The asterisks denote the centres of chirality.*

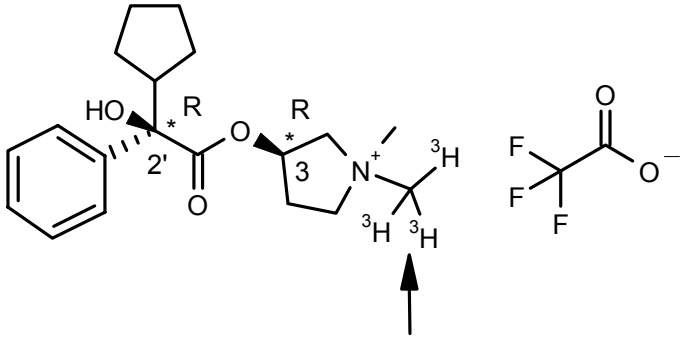
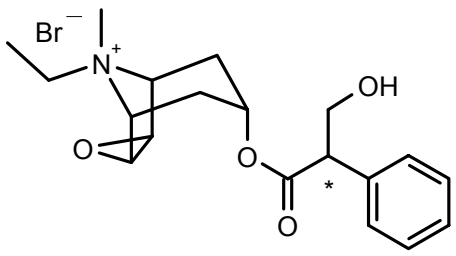
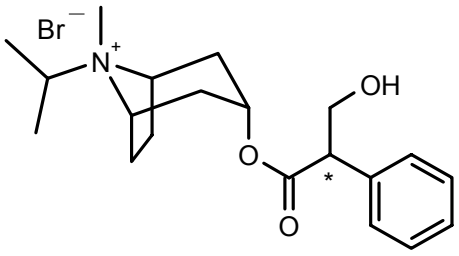


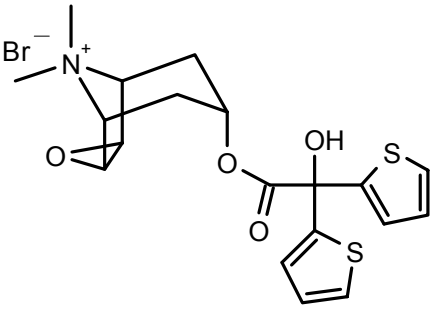
Table 2.21 *Structures of the most important approved drugs for use in COPD therapy. The asterisks denote the centres of chirality.*



Oxitropium



Ipratropium



Tiotropium

2.7 M_2-selective antagonists related to dimethindene

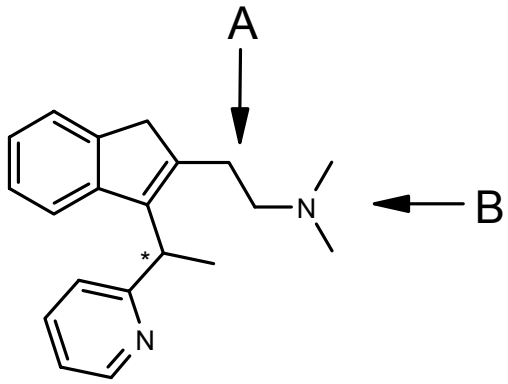
Considerable results were achieved at many pharmacological targets but up to now only little advance was made in the development of selective muscarinic antagonists. M_2-selective antagonists are of great interest for several therapeutic indications, e.g. in therapy of AD. Even more promising than a therapeutic aspect in AD is the idea to develop a highly potent, M_2-selective, brain-penetrating compound for PET-studies. A compound with this properties could be used as a diagnostic tool to verify AD diagnosis *in vivo* as the total number of M_2 receptor decreases with proceeding of AD (Petrella et al., 2003). This would be a mile-stone, as only post-mortem studies can provide a 100% safe AD diagnosis to date.

Dimethindene (**57A**) is used therapeutically since many years as a histamine H_1 receptor antagonist. It is well known that this drug is capable to cross the blood brain barrier causing sedation as a side effect in human therapy. **57A** is currently used as a racemic mixture in therapy. In several functional models a 50 - 1000-fold higher affinity was found for the (R)-(-)-configurated enantiomer of dimethindene to H_1 receptors in comparison to the (S)-(+)-configurated enantiomer (Borchard et al., 1985). These findings were confirmed in binding studies, too (Ter Laak et al., 1993). It was shown that drowsiness following application of one of the enantiomers to humans was related to the (R)-(-)-configurated isomer (Nicholson et al., 1991) as a result of its high H_1 affinity. In functional studies in our laboratories at $M_{1\text{-}3}$ receptors and in binding studies at native $M_{1\text{-}4}$ subtypes the inverse effect was seen at muscarinic receptors where the (S)-(+)-configurated enantiomer showed higher affinities (Pfaff et al., 1995). These binding studies revealed that (+)-dimethindene had an at least 5-fold higher affinity at M_2 versus $M_{1,3,4}$. However, affinity to H_1 receptors was almost as high as affinity to M_2. Taking **57A** as a starting point, considerable efforts were made in our laboratory to improve absolute affinity and subtype selectivity at muscarinic M_2 receptors and at the same time to reduce affinity to histamine H_1 receptors. This extensively conducted research culminated in an analogue possessing two isopropyl substituents (**75A**) instead of two methyl groups at the basic nitrogen (Böhme et al., 2003). The (+)-enantiomer had an at least 36-fold selectivity for M_2 versus the other subtypes and a 58-fold specificity to H_1. However, one aim was still not reached - affinity at M_2 was not increased (pK_i = 7.37) in comparison to the parent compound.

Before synthesis for this study was started, a complete SAR study of the already existing dimethindene derivatives was done at hH_1 receptors. After a thorough examination of binding data at M_{1-5} and H_1, the hypothesis was postulated that reducing the side chain length connecting the amino moiety to the indene structure would increase affinity to muscarinic receptors and simultaneously decrease H_1 affinity. Synthesis was directed in a direction to prove this working thesis. All tested analogues were chiral compounds. Unless explicitly expressed, all drugs were tested as racemic mixtures.

Table 2.22 shows the formula of dimethindene (**57A**) which served as a starting point in this SAR study with arrows indicating the general sites of modifications.

Table 2.22 *Structure of dimethindene (**57A**) and general sites of structural variations. The asterisk denotes the centre of chirality.*



A Modification in side chain length

B Changes in substituents at the basic amino nitrogen

The following molecule variations were testes in our studies:

- Derivatives with modifications in N-alkylation pattern and modification in side chain length. Table 2.23 gives the structures. Compounds having the letter "**A**" affixed to the compound number possess a side chain equal to the parent compound **57A** (n=2). Analogues with a "**B**" attached to the number have a shortened side chain (n=1). Derivatives **62** and **63** were only tested with reduced chain length ("**B**").

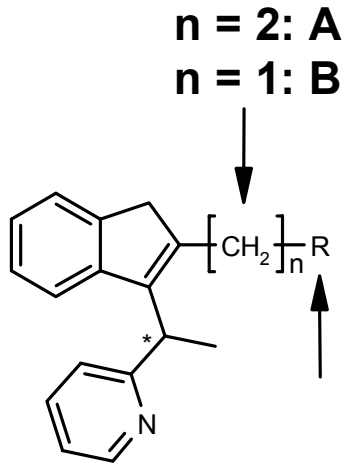
- A series of compounds with either a benzyl or a phenylethyl substituent attached to the basic nitrogen with reduced side chain length, **65B** - **74B**, was tested. Table 2.24 displays the compounds.

- Three compounds were separated in enantiomers. These were the parent compound **(+/-)-57A**, derivative **(+/-)-72B** and the diisopropyl analogue **(+/-)-75A**. Structure of **75A** is given in Table 2.25.

- Analogues **76B** and **77B** with reduced chain length closely related to **72B** were examined. Structures are given in Table 2.26.

- Three sets of compounds with insertion of a para- or meta-substituent (**78B** - **83B**) in the phenylethyl substituent of **72B** were tested. Structures are shown in Table 2.27.

Table 2.23 *Compounds with modifications in side chain length and N-alkylation. The asterisk denotes the centre of chirality.*



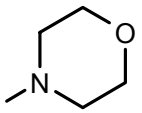
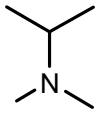
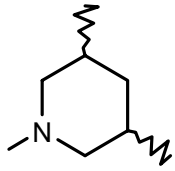
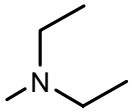
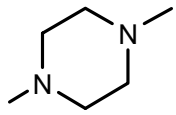
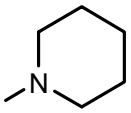
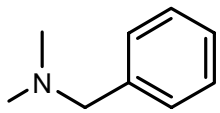
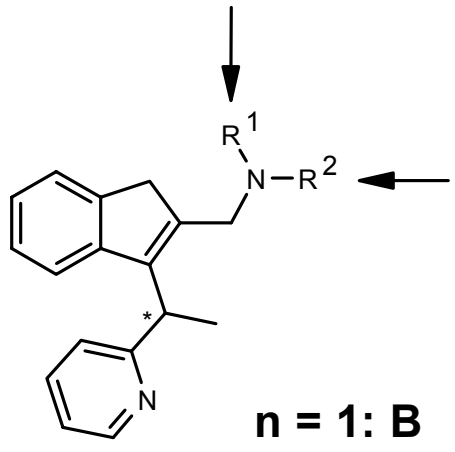
No.	R	No.	R
57A/B		61A/B	
58A/B		62B	
59A/B		63B	
60A/B		64A/B	

Table 2.24 *Compounds with a benzyl or phenylethyl substituent at the basic nitrogen and reduced side chain length. The asterisk denotes the centre of chirality.*



n = 1: B

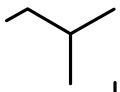
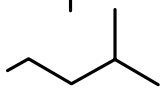
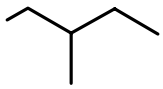
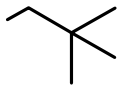
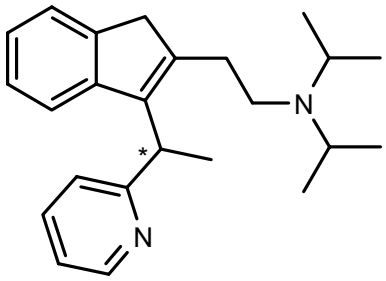
R^1 Benzyl	R^1 Phenylethyl	
No.	**No.**	**R^2**
65B	**70B**	H
64B	**-**	CH_3
-	**71B**	C_2H_5
66B	**72B**	
67B	**-**	
68B	**-**	
-	**73B**	
69B	**74B**	

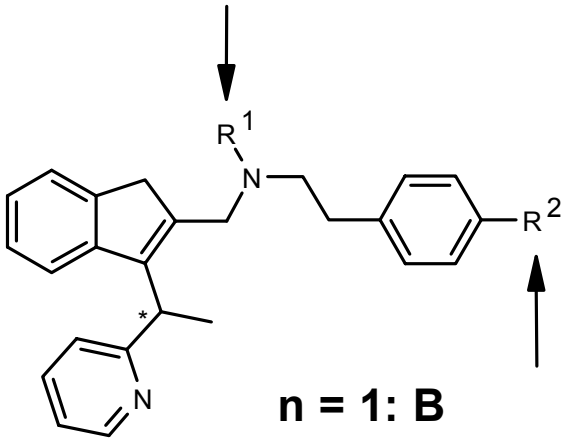
Table 2.25 *Structure of compound **75A**. The asterisk denotes the centre of chirality.*



n = 2: A

No. (+/-)-75A

Table 2.26 *Structure of compounds closely related to **72B**. The asterisk denotes the centre of chirality.*



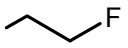
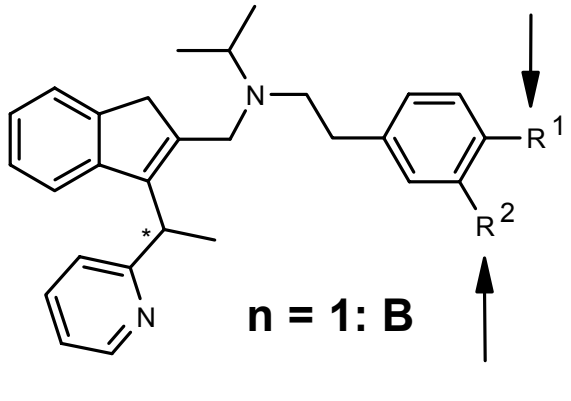
No.	R 1	R 2
76B		H
77B		CH_3

Table 2.27 *Structure of compounds closely related to **72B** with insertion of p- or m-substituents at the phenyl ring. The asterisk denotes the centre of chirality.*



No.	R 1	R 2
78B	H	OCH_3
79B	OCH_3	H
80B	H	Cl
81B	Cl	H
82B	H	F
83B	F	H

3 Material and Methods

3.1 Commercially available drugs

3.1.1 Reference substances

4-DAMP (Tocris, Bristol, UK)
Astemizole (Promochem, Wesel, Germany)
Atropine sulfate (E. Merck, Darmstadt, Germany)
Cetirizine dihydrochloride (Promochem, Wesel, Germany)
(R/S)-Chlorpheniramine maleate (Sigma / RBI, Deisenhofen, Germany)
Diphenhydramine hydrochloride (Sigma / RBI, Deisenhofen, Germany)
GR113808 (Tocris, Bristol, UK)
McN-A-343 chloride (Sigma / RBI, Deisenhofen, Germany)
MDL72222 (Bemesetron) (Sigma / RBI, Deisenhofen, Germany)
Mepyramine maleate (Sigma / RBI, Deisenhofen, Germany)
Metoclopramide hydrochloride (Sigma / RBI, Deisenhofen, Germany)
RS23597-190 hydrochloride (Tocris, Bristol, UK)
RS39604 hydrochloride (Tocris, Bristol, UK)
SB203186 hydrochloride (Tocris, Bristol, UK)
t-Triprolidin hydrochloride (Sigma / RBI, Deisenhofen, Germany)
Tropisetron (Sigma / RBI, Deisenhofen, Germany)
Terfenadine (Sigma / RBI, Deisenhofen, Germany)

3.1.2 Radiochemicals

Amersham, Buckinghamshire, England:
1-[N-methyl-^{3}H]scopolamine methylchloride (SA 84.0 Ci/mmol) = [^{3}H]NMS
[N-methyl-^{3}H]GR113808 (SA 83.0 Ci/mmol)
[N-methyl-^{3}H](3R, 2'R)-glycopyrronium ([^{3}H]43D) (SA 84.0 Ci/mmol) was synthesised starting from a cold precursor provided by M. Walter, Vienna, Austria.
[Pyridinyl-5-^{3}H]pyrilamine (SA 28.0 Ci/mmol) = [^{3}H]mepyramine

Perkin Elmer / NEN, Bosten, MA, USA:
1-[N-methyl-^{3}H]scopolamine methylchloride (SA 78.0 Ci/mmol) = [^{3}H]NMS
[N-methyl-^{3}H]GR 65630 (SA 75.5 Ci/mmol)

3.1.3 Buffer compounds and solvents

E. Merck, Darmstadt, Germany:

N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid sodium salt (HEPES)

Tris(hydroxymethyl)-aminomethane (Tris)

$MgCl_2$ x $6H_20$

NaCl

0.1 M-hydrochloric acid

1 M-sodium hydroxide

Dimethylsulfoxide (DMSO)

Methanol

3.1.4 Material for cell culture and protein assay

Bovine serum albumine standard (Sigma / RBI, Deisenhofen, Germany)

Cell culture dishes (Nunc, Roskilde, Denmark)

Dulbecco's modified Eagle's medium (BioWhitaker Inc., Walkersville, MD, USA)

Fetal calf serum (Gibco, Grand Island, NY, USA)

Glutamine (BioWhitaker Inc., Walkersville, MD, USA)

Lowry - protein assay kit (Bio-Rad, Richmond, USA)

Non-essential amino acids (BioWhitaker Inc., Walkersville, MD, USA)

Phosphate buffered saline (BioWhitaker Inc., Walkersville, MD, USA)

Penicillin G / streptomycin mixture (BioWhitaker Inc., Walkersville, MD, USA)

Trypsin-EDTA (BioWhitaker Inc., Walkersville, MD, USA)

3.1.5 Material for radioligand binding assays

Lumasafe Plus scintillation cocktail (Packard Bioscience, Dreieich, Germany)

Polyethylene imine (50% in purified water) (Sigma / RBI, Deisenhofen, Germany)

Whatman GF/B glas fibre filter (A. Hassel, München, Germany)

Zinsser mini scintillation vials (Zinsser Analytik, Frankfurt, Germany)

3.2 Gifts

3.2.1 Reference substances

Alosetron hydrochloride (Prof. S. Elz, Regensburg, Germany)
Dimethindene ((±)-maleate, (+/-)-tartrates) (Dr. D. Rehn, Zyma, München, Germany)
Dolasetron mesylate (Prof. S. Elz, Regensburg, Germany)
Fexofenadine hydrochloride (Prof. S. Elz, Regensburg, Germany)
Granisetron hydrochloride (Prof. S. Elz, Regensburg, Germany)
HHSiD hydrochloride (Prof. R. Tacke, Würzburg, Germany)
Himbacine hydrochloride (Dr. W. C. Taylor, Sydney, Australia)
Ipratropium bromide (Thomae, Biberach/Riss, Germany)
Ondansetron hydrochloride (Prof. S. Elz, Regensburg, Germany)
PD102807 (Dr. R. D. Schwarz, Parke-Davis, Ann Arbor, USA)
Pirenzepine hydrochloride (Thomae, Biberach/Riss, Germany)

3.2.2 Cells

CHO-K1 cells stably expressing hH_1 receptors (Prof. R. Leurs, Amsterdam, NL)
CHO-K1 cells stably expressing $hM_{1\text{-}5}$ receptors (Dr. J. Wess, NIDDK, National Institutes of Health, Bethesda, MD, USA)
Membranes from HEK293 cells stably expressing h5-HT_{3A} receptors (PD M. Brüss, Bonn, Germany)
Membranes from HEK293 cells stably expressing h5-$HT_{4(b)}$ receptors (PD M. Brüss, Bonn, Germany)

3.3 Synthesis

- Analogues of ondansetron and metoclopramide were synthesised in the group of Prof. Dr. S. Elz, Institute of Pharmaceutical Chemistry, University of Regensburg, Germany.

- Analogues of glycopyrrolate were synthesised in the group of Prof. Dr. C. Noe, Institute of Pharmaceutical Chemistry, University of Vienna, Austria.

- Analogues of dimethindene were synthesised in the group of Prof. Dr. G. Dannhardt, Institute of Pharmaceutical Chemistry, University of Mainz, Germany.

- Analogues of McN-A-343 were synthesised in our laboratories by Dr. U. Moser and U. Hermanni.

3.4 Anions of compounds

Many compounds were obtained as free bases, those obtained as salts are listed below in Table 3.1 next to their corresponding anions.

Table 3.1 *Anions of compounds.*

chloride:	**1**
bromide:	**43**-**56**
iodide:	**11**, **31**, **32**$^{+}$, **(R/S)-33**$^{+}$, **34**$^{+}$, **(±)-38**$^{+}$ - **(±)-42**$^{+}$
perchorate:	**(R/S)-35**$^{+}$
oxalate:	**32**, **(R/S)-33**, **34**, **(R/S)-35** - **(R/S)-37**, **(±)-38** - **(±)-42**
tosylate:	**(R/S)-36**$^{+}$, **(R/S)-37**$^{+}$, **(±)-40**$^{+}$, **65B**, **(±)-72B**
maleate:	**17**, **(±)-57B**, **58A** - **61A**

3.5 Chirality

Within the series of tested compounds were several chiral molecules. The pure enantiomers of compounds **72B** and **75A** were separated via HPLC by Dr. T. Böhme, Aventis, Frankfurt, Germany, and had no detectable impurities (for detailed method see Böhme et al., 2003). For compounds related to metoclopramide, ondansetron and McN-A-343, stereochemical purity was high according to pharmacological results.

3.6 Stock solutions

To make 1 mM stock solutions small amounts of DMSO, methanol and 0.1 M hydrochloric acid were used to dissolve compounds when necessary, before filling up to the required volume with demineralised water. The radioligand dilution was prepared with demineralised water.

3.7 Preparation of buffers

Buffers were made of analytical grade purity chemicals:

HEPES buffer:	HEPES	20 mM
	$MgCl_2$	10 mM
	NaCl	100 mM
	NaOH	ad pH 7.4
Tris buffer:	Tris	5 mM
	HCl	ad pH 7.4

3.8 Methods

3.8.1 Cell culture

CHO-K1 cells stably transfected with $hM_{1\text{-}5}$ or hH_1 receptors were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 I.U./ml penicillin G, 100 µg/ml streptomycin, 2 mM glutamine and 0.1 mM non-essantial amino acids in a humidified atmosphere with 5-10% CO_2 in monolayer culture. 1 ml-stocks were quickly thawed in a 37°C water bath and suspended in approximately 35 ml of warmed growth medium in a 175 cm^2 cell culture flask. The next day approximately two thirds of the medium were replaced. For passage (done at confluence three times weekly) the whole medium was removed, cells were washed with 37°C phosphate buffered saline (PBS), loosened with 2 ml trypsin-EDTA and resuspended in fresh medium. According to cell density a splitting ratio of 1:4 to 1:8 was used. To obtain stock aliquots, washed and trypsinised cells were

collected by centrifugation (10 min, approximately 300 g), resuspended in growth medium supplied with 10% DMSO and slowly frozen to -80°C before storage in liquid nitrogen. One 175 cm^2 cell culture bottle was used to obtain three stock aliquots.

3.8.2 Membrane preparation

Membranes of CHO-K1 cells were prepared four passages after thaw. For harvesting, cells were put in 525 cm^2 culture dishes after the last splitting. At 80-100% confluence cells were washed with ice-cold PBS, scraped into ice-cold HEPES buffer and homogenised with ultrasonic waves using a Branson Cell Disruptor B15 (duty cycle 20%, output control setting 3, 45 seconds) Danbury, CT, USA. Membranes were pelleted at 30.600g for 30 min at 4°C, resuspended in HEPES buffer using a hand potter, aliquoted and stored in liquid nitrogen. The protein concentration was determined as written below. The aliquots were stored in liquid nitrogen. During storage no change in binding parameters appeared.

3.8.3 Protein assay

Protein concentration was determined according to the method of Lowry (Lowry et al., 1951) using a Bio-Rad protein assay kit in 5 mM Tris-buffer pH 7.4 and bovine serum albumin as standard. As HEPES disturbs this assay, membranes were pelleted and washed twice with Tris buffer before protein concentration was determined.

3.8.4 Radioligand binding studies

Radioligand binding assays were carried out according to literature (examples are given in Table 3.2) with the following modifications. All assays were carried out on 96-well plates (maximum volume 1.2 ml) in a total volume of 1 ml in HEPES buffer pH 7.4. Final protein concentrations (in µg/ml) were: M_1: 5; M_2: 20; M_3: 8; M_4: 7; M_5: 13; H_1: 27; 5-HT_3: 5; 5-HT_4: 23. These assay conditions were derived from the total number of receptors, the radioligand equilibrium dissociation constant (K_D), which were both measured in saturation binding experiments, and the rule that receptor concentration should be less than 10% of the radioligand K_D (Hulme, 1992).

Incubation was carried out at 25°C in a slowly stirred waterbath and terminated by vacuum filtration through a Brandell cell harvester (Brandell, Gaithersburgh, MD, USA) onto Whatman GF/B filters, presoaked in 0.5% polyethylene imine solution. Filters were washed three times with 1 ml ice-cold binding buffer and transferred into scintillation vials, 3 ml of scintillant were added and shaken for 2 hours. Samples were counted in a Wallac ß-counter (Wallac and Berthold, Milton Keynes, UK). The counting efficiency was approximately 53%.

Table 3.2 *Used radioligands and concentrations at corresponding receptors. Drugs and their final concentrations to determine non-specific binding (NSB). Literature examples concerning the assays are given in the last column.*

Receptor	Radioligand	c [nM]	NSB	c [µM]	Literature
M_{1-5}	[^{3}H]NMS	0.2	atropine	1	Dörje et al., 1991 Buckley et al., 1989
H_1	[^{3}H]mepyramine	1.0	terfenadine	100	Ter Laak et al., 1993 Smit et al., 1996
5-HT_3	[^{3}H]GR65630	0.1	tropisetron	1	Sharif et al., 1991 Miyake et al., 1995
5-HT_4	[^{3}H]GR113808	0.1	RS39604	1	Pindon et al., 2002 Blondel et al., 1998
M_{1-5}	[^{3}H]43D	0.2	atropine	1	This work

3.8.5 Saturation binding experiments

To determine the total number of receptors (B_{max}) in the membrane preparation and the equilibrium dissociation constant (K_D) of the radioligand at the corresponding subtype, saturation binding experiment were carried out. Each new membrane batch was tested to make sure that the binding parameters did not change over time. 10 increasing concentrations of radioligand (spanning approximately three orders of magnitude up to a maximum of 100 times K_D where possible) were used to determine the K_D. Incubation time was 2 h with exception for determination of the K_D of [^{3}H]43D at M_3 receptors where 4 h were chosen due to the slow dissociation of the radioligand at this subtype, to ensure that equilibrium was reached. Non-specific

binding (NSB) was determined according to Table 3.2. Each experiment was carried out in triplicate with the following sample conditions:

- 600 µl of HEPES buffer (to determine total binding) or
  500µl of buffer (to determine NSB)
- 100 µl of a suitable drug given in Table 3.2 (to determine NSB)
- 100 µl of radioligand dilution
- 300 µl of membrane suspension to initiate incubation.

The data were analysed as written in 3.9.1.

3.8.6 Competition binding experiments

To determine the inhibition constant K_i, representing the binding affinity of unlabelled test compounds, competition experiments were carried out. A single concentration of radioligand (given in Table 3.2) was used together with 7 - 9 different amounts of the test compound acting as a competitor. The radioligand concentration in competition experiments is recommended to be in range of the radioligand's K_D (Hulme, 1992). In combination with a reasonable concentration of binding sites, depletion of free radioligand can be avoided and low non-specific binding is achieved, resulting in a good "signal-to-noise-ratio". [^{3}H]43D was used at a concentration of 0.2 nM which is higher than K_D. This was done in order to reach equilibrium at all subtypes in an incubation time of 4.5 h. Incubation time was 2 h for all other competition experiments.

The competition binding experiments were carried out in duplicate with the following sample composition:

- 100 µl of test compound dilution (competition sample) or
  100 µl of a suitable drug dilution given in Table 3.2 (to determine NSB)
- 500 µl of HEPES buffer (competition samples and NSB) or
- 600 µl of HEPES buffer (to determine total binding)
- 100 µl of radioligand dilution
- 300 µl of membrane suspension to initiate incubation.

The data were analysed as written in 3.9.2.

3.8.7 Kinetic binding experiments

3.8.7.1 Association binding experiments

To obtain the observed rate constant (k_{obs}), association rate constant (k_{on}) and the dissociation rate constant (k_{off}), association curves with 12 points of time were recorded with 6 - 8 different concentrations of [^{3}H]43D (Motulsky, 1999). Concentrations were spanning approximately one order of magnitude (Table 3.3). Maximum incubation time was 2 h with exception of M_3 receptor (4 h).

Table 3.3 *Concentrations of [^{3}H]43D in association binding experiments.*

Subtype	[^{3}H]43D [nM]							
M_1	0.10	0.20	0.30	0.40	0.50	0.60	0.80	1.00
M_2	0.05	0.10	0.20	0.30	0.40	0.60	0.80	1.00
M_3	0.08	0.12	0.16	0.20	0.25	0.30	0.35	0.45
M_4	0.08	0.12	0.16	0.20	0.25	0.30	0.35	0.45
M_5	0.10	0.15	0.20	0.25	0.30	0.40	0.50	0.60

The association binding experiments were carried out in duplicate with the following sample composition:

- 600 µl of HEPES buffer (kinetic samples) or
  500 µl of HEPES buffer (to determine NSB)
- 100 µl of 10 µM atropine solution (to determine NSB)
- 100 µl of radioligand dilution
- 300 µl of membrane suspension to initiate incubation.

The obtained data were analysed as described in 3.9.3.

3.8.7.2 Dissociation binding experiments

To separately determine the dissociation rate constant (k_{off}) of [^{3}H]43D at the M_1 and M_2 receptor subtypes, off-kinetic experiments were carried out. The radioligand concentration was 0.2 nM and preincubation time with the membrane to reach equilibrium was 1 h. Then atropine was added at 24 points of time to block further

association. Maximum incubation time after adding atropine was 4 h in the case of M_1 and 2 h in the case of M_2.

3.9 Data analysis and statistics

Data were analysed by curve fitting procedures using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA, USA). Before fitting, specific binding was calculated for each point by subtracting the non-specific binding from the measured total binding.

3.9.1 Saturation binding experiments

3.9.1.1 One-site binding model

Data from saturation binding experiments were fitted to the equation

(1) $$y = \frac{B_{\max} \cdot x}{(K_D + x)}$$

to determine the equilibrium dissociation constant (K_D) of a radioligand and the total amount of binding sites (B_{max} given in CPM). X is the concentration of radioligand and y is the amount of specific bound radioactivity (in CPM) calculated by subtraction of NSB from total binding.

Measured CPM values were converted into real concentration values [nM] according to equation:

(2) $$c = \frac{CPM \cdot e}{2220 \cdot V \cdot SA}$$

with e displaying the counting efficacy factor (1.883), V = assay volume and SA = specific activity of the radioligand [Ci/mmol] (depending on kind and batch of ligand).

3.9.1.2 Two-site binding model

Data from saturation binding experiments of [^{3}H]mepyramine were also fitted to a two-site binding model

(3) $$y = \frac{B_{\max 1} \cdot x}{(K_{D1} + x)} + \frac{B_{\max 2} \cdot x}{(K_{D2} + x)}$$

The fits (1) and (3) were compared with an F-test.

3.9.2 Competition binding experiments

Data from competition binding experiments were fitted to the equation

(4) $$y = \frac{B_0}{1 + 10^{(\log IC_{50} - x) \cdot n_H}}$$

to compute Hill coefficient (n_H), and to equation

(5) $$y = \frac{B_0}{1 + 10^{x - \log IC_{50}}}$$

to calculate the IC_{50} value of a cold competitor. X is the logarithm of the competitor concentration and y is the specific bound radioactivity in CPM. B_0 is the amount of specific radioligand binding in an experiment when no competitor is present (or its concentration is infinitesimal).

K_i values of the test compounds were derived from IC_{50} values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973),

(6) $$K_i = \frac{IC_{50}}{(1 + \frac{L}{K_D})}$$

with L = concentration of radioligand, K_D = equilibrium dissociation constant of the radioligand as the following conditions were provided:

- No ligand depletion has occurred.
- The experiment has reached equilibrium.
- Binding was fully reversible and follows the law of mass action with a Hill coefficient not different from unity.
- The receptors were homogenous and all binding sites had the same affinity for the ligand.
- There was no cooperativity-binding, ligand-ligand interaction or chemical transformation.

3.9.2.1 Two-site binding model

If the obtained Hill coefficient was significantly different from unity the competition curves were additionally fitted to a to two-site binding model.

$$(7) \qquad y = \frac{B_0 \cdot fraction1}{1+10^{(x-\log IC_{50\_1})}} + \frac{B_0 \cdot (1-fraction1)}{1+10^{(x-\log IC_{50\_2})}}$$

This equation describes the competition of a ligand for two types of receptors. The radioligand has identical affinities for both receptors, but the competitor has a different affinity for each. Fraction1 is the fraction of the receptors that have an affinity described by $\log IC_{50\_1}$. The remainder of the receptors have an affinity described by $\log IC_{50\_2}$. If $\log IC_{50\_1}$ is smaller than $\log IC_{50\_2}$, then fraction_1 is the fraction of high affinity sites.
The fits (5) and (7) were compared with an F-test.

3.9.3 Kinetic binding experiments

3.9.3.1 Association binding experiments

To calculate the radioligand's association rate constant (k_{on}) and dissociation rate constant (k_{off}) out of the observed rate constant (k_{obs}) and the concentration of the radioligand (c_{RL}) the following steps were performed (Motulsky, 1999):

To calculate k_{obs} from the association curves the following formula was used for a non-linear curve fitting procedure:

$$(8) \qquad y = y_{\max} \cdot (1-e^{-k_{obs} \cdot t})$$

where y_{max} equals the maximum binding in CPM at equilibrium for a special concentration of radioligand (which is different from B_{max}), t = time in minutes and k_{obs} = observed rate constant [min^{-1}].
The rearrangement of formula

$$(9) \qquad k_{on} = \frac{k_{obs} - k_{off}}{c_{RL}}$$

leads to

(10) $$k_{obs} = k_{on} \cdot c_{RL} + k_{off}$$

which can be fitted as a straight line in a linear regression to

(11) $$y = m \cdot x + c$$

in which x = c_{RL} [nM], y = k_{obs} [min^{-1}], m = k_{on} [nM^{-1} min^{-1}] and c = k_{off} [min^{-1}] (Anthes et al., 2002).

The kinetic K_D ($kinK_D$) - in contrast to the saturation K_D ($satK_D$), which was assessed in saturation binding assays - was calculated by the formula

(12) $$kinK_D = \frac{k_{off}}{k_{on}}$$

using k_{on} and k_{off} values determined in the association experiments in the case of $M_{3,4,5}$ receptors. For the M_1 and M_2 receptor the k_{off} value to calculate the kinetic K_D was taken from the separately done dissociation experiments (see below). For the M_1 receptor the k_{on} value was computed as an average out of the k_{obs} values from the association curves using the off-experiments k_{off} and the used radioligand concentration.

3.9.3.2 Dissociation binding experiments

To ensure the validity of the data obtained in the association binding experiments, dissociation binding experiments were carried out at M_1 and M_2 receptor to directly assess k_{off}. Non-linear curve fitting was used for the following formula displaying a classical first order decay process.

(13) $$TotalBinding = NSB + SpecificBinding \cdot e^{-k_{off} \cdot t}$$

where t = time in minutes, k_{off} = dissociation rate constant [min^{-1}]. Total binding was in CPM and NSB was fitted by non-linear regression.

The dissociation half life ($t_{1/2}$) of [^{3}H]43D was calculated by

(14) $$t_{1/2} = \frac{\ln 2}{k_{off}}$$

where k_{off} was used in [min^{-1}] and $t_{1/2}$ was calculated in minutes.

3.10 Statistics

Unless stated otherwise, data are presented as means with standard deviation (±SD) of at least three independent experiments. Competition binding experiments and kinetic binding experiments were carried out in duplicate, saturation binding experiments in triplicate each. Statistical significance was assessed using Student's t-test with $p<0.05$. The F-Test was used to compare the non-linear regression fits for the one- and two-site binding models (see 3.9.1.2 and 3.9.2.1).

4 Results

4.1 Validation of assays and general considerations

4.1.1 Saturation binding experiments

K_D and B_{max} values obtained were summarized in Table 4.1. In muscarinic and serotonergic saturation binding experiments specific binding was saturable and consistent with a one-site binding model. For all tested subtypes non-specific binding increased linearly within the range of the used concentrations of radioligand. Scatchard plots were linear indicating a one-site binding model without co-operativity. At H_1 receptors, binding data fitted better to a two-site model.

4.1.1.1 Muscarinic M_{1-5} receptors

Results obtained with [^{3}H]NMS at M_{1-5} receptors were in good agreement with data obtained previously in our laboratory and literature (Dörje et al., 1991). The mean K_D values obtained in a huge number of experiments, carried out in our laboratory by Dr. C. Keim and K. Kreutzmann, were used for further calculations. As an example the binding isotherm for the M_1 subtype is shown in Fig. 4.1.

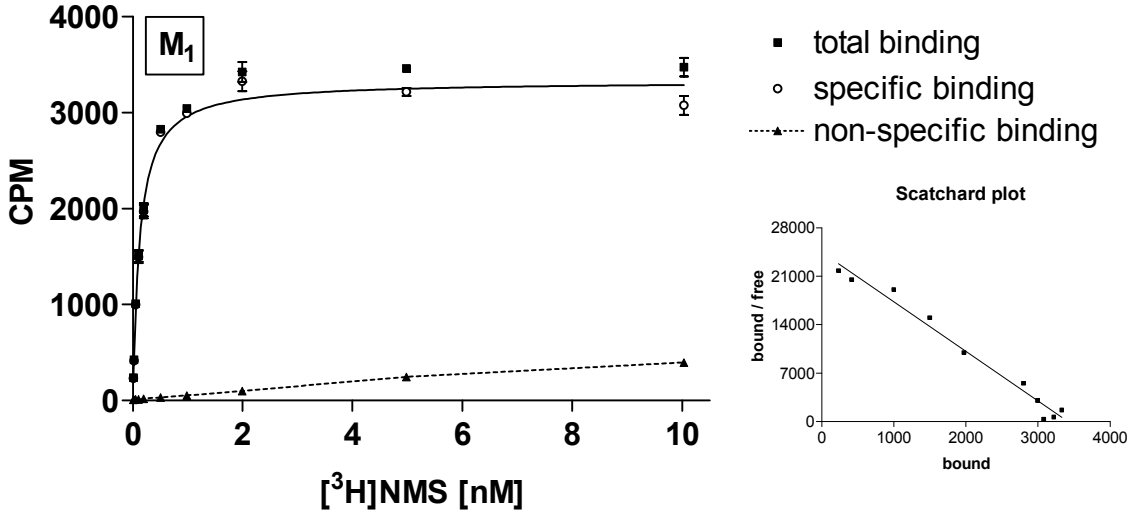


Fig. 4.1 *Saturation binding isotherms displaying total (boxes), specific (circles) and non-specific binding (triangles) of [^{3}H]NMS to cloned hM_{1-5} receptors stably expressed in CHO-K1 cells and the corresponding Scatchard plot. Data represent one typical experiment each.*

4.1.1.2 Serotonin 5-HT_{3A} receptors

The saturation binding isotherm of [^{3}H]GR65630 at the 5-HT_{3A} subtype is given in Fig. 4.2. The calculated K_D value is close to several literature values. Our value is similar by a factor of 2 - 4 to data obtained in binding studies with rat tissue preparations (Kilpatrick et al., 1987, 1989). Similar results were reported for mouse 5-HT_3 receptors stably expressed in CHO cells (Hovius et al., 1998) and a study with bovine brain tissue (Miller et al., 1992). Great differences to other studies using HEK293 cells stably expressing h5-HT_{3A} receptors (Brüss et al., 1999) exist. A discrepancy of approximately factor 20 between our value and this literature K_D is surprising at the first glance. But when comparing the assays, major differences between the used assay conditions (buffer, pH, temperature and incubation time) are obvious, and may explain the differences in the resulting K_D values.

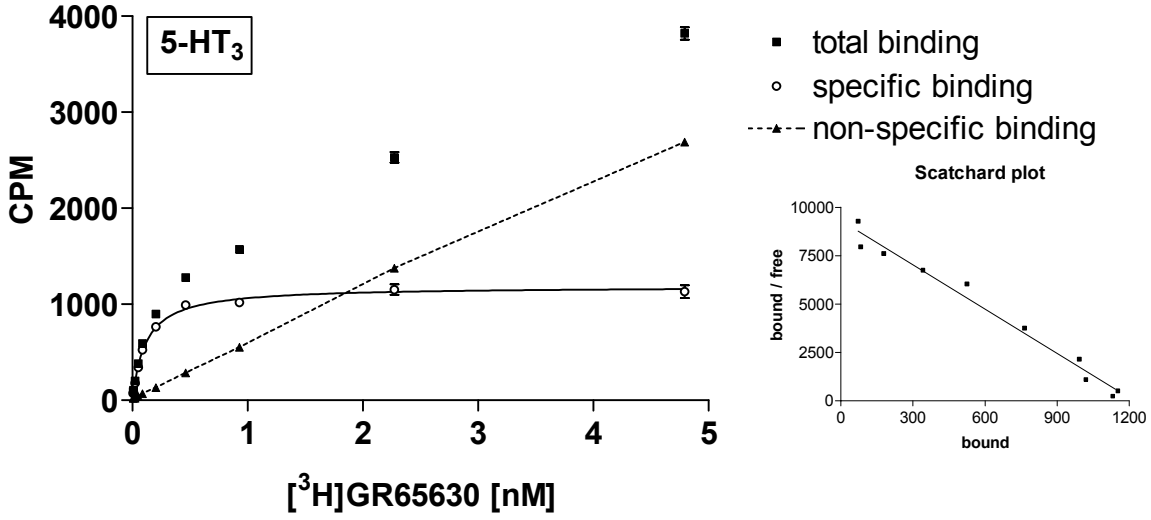


Fig. 4.2 *Saturation binding isotherms displaying total (boxes), specific (circles) and non-specific binding (triangles) of [^{3}H]GR65630 to cloned h5-HT_{3A} receptors stably expressed in HEK293 cells and the corresponding Scatchard plot. Data represent one typical experiment each.*

4.1.1.3 Serotonin 5-$HT_{4(b)}$ receptors

Fig. 4.3 displays the saturation binding isotherm of [^{3}H]GR113808 at the 5-$HT_{4(b)}$ subtype. The K_D value given in Table 4.1 is in good agreement with literature results using [^{3}H]GR113808 as radioligand. Very good agreement exists to a study using HEK293 cells stably expressing h5-$HT_{4(b)}$ (Pindon et al., 2002) and a study using

human cortex preparations (Wong et al., 1996). However, a discrepancy of factor 8 exists to a study using transiently expression of h5-$HT_{4(b)}$ in COS-7 cells (Blondel et al., 1998). As assay conditions were similar, it is likely that this difference results from the different expression systems.

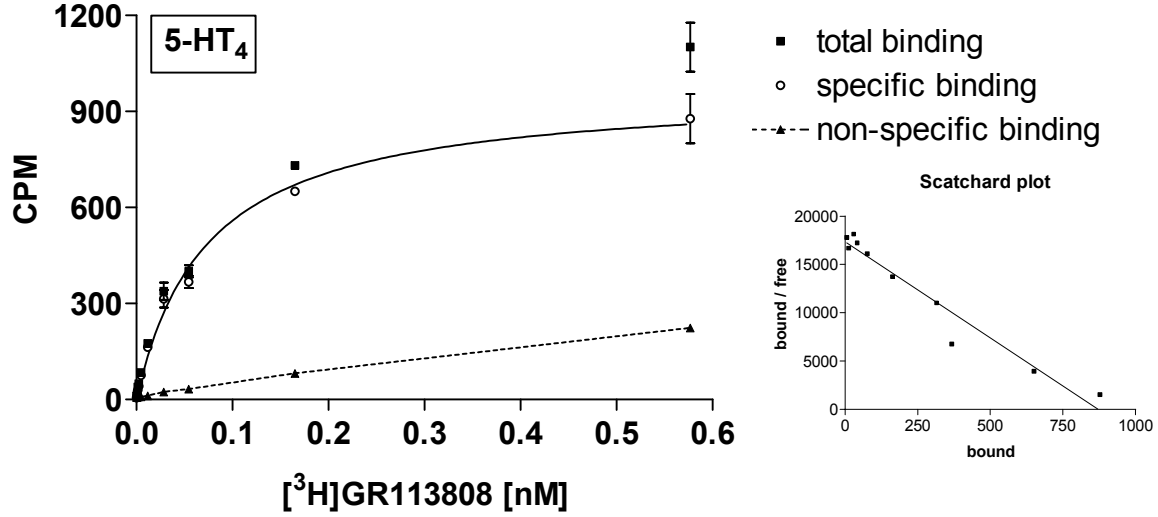


Fig. 4.3 *Saturation binding isotherms displaying total (boxes), specific (circles) and non-specific binding (triangles) of [^{3}H]GR113808 to cloned h5-$HT_{4(b)}$ receptors stably expressed in HEK293 cells and the corresponding Scatchard plot. Data represent one typical experiment each.*

4.1.1.4 Histamine H_1 receptors

Saturation binding curves of [^{3}H]mepyramine at H_1 receptors fitted significantly better to a two-site binding model. The second, low-affinity binding site, could not be fitted well (as the concentration of radioligand could not be increased further due to financial constraints), but was at least two orders of magnitude lower in affinity than the high-affinity site. In Fig. 4.4 the fits for a one- and a two-site binding model for [^{3}H]mepyramine are given. Scatchard plots were curve-linear, indicating a second binding site, too. For further competition studies the high-affinity site was assumed to be the H_1 receptor. The obtained K_D value for [^{3}H]mepyramine (Table 4.1) at the hH_1 receptor is in good agreement with literature. Data are similar to previous studies with CHO-K1 cells stably expressing hH_1 receptors (Smit et al., 1996; Anthes et al., 2002). A difference of factor 4.5 exists to the K_D reported by Moguilevsky et al. (1994). However, very different assay conditions were used in that publication. Good

agreement exists to saturation binding studies in GPI preparations (Ter Laak et al., 1993), compared to our results.

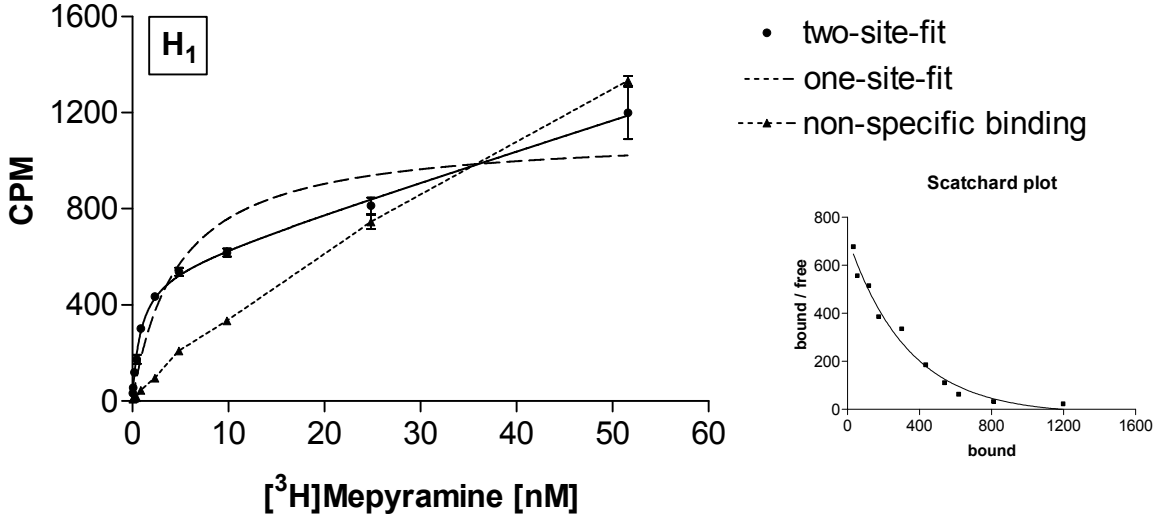


Fig. 4.4 *Saturation binding isotherms displaying the fit of specific binding (circles) to a two-site model (solid line), a one-site model (dashed line) and non-specific binding (triangles) of [^{3}H]mepyramine to cloned hH_1 receptors stably expressed in CHO-K1 cells and the corresponding Scatchard plot. Data represent one typical experiment each.*

Table 4.1 *Equilibrium dissociation constants (K_D) of the used radioligands (compare to Table 3.2) at their corresponding receptors and the total number of specific binding sites (B_{max}) in membranes from stably transfected cells derived from saturation experiments.*

Receptor	K_D [nM]	pK_D	B_{max} [fmol/µg protein]
M_1	0.19 [a)]	9.72	4.11 ± 0.50
M_2	0.33 [a)]	9.48	0.56 ± 0.04
M_3	0.17 [a)]	9.77	3.24 ± 0.06
M_4	0.10 [a)]	10.00	1.25 ± 0.10
M_5	0.48 [a)]	9.32	0.45 ± 0.03
H_1	0.84 ± 0.07	9.06 ± 0.03	0.73 ± 0.10
5-HT_{3A}	0.13 ± 0.05	9.91 ± 0.15	2.78 ± 0.57
5-$HT_{4(b)}$	0.078 ± 0.005	10.11 ± 0.03	0.39 ± 0.00

[a)] The mean K_D values at muscarinic subtypes were taken from former experiments carried out by Dr. C. Keim in our laboratory (Böhme et al., 2003).

4.1.2 Competition binding experiments

At all receptor subtypes a variety of reference substances was tested in competition studies to demonstrate the validity of the binding assays in comparison to data reported in literature. Representative competition curves are given for each assay. Unless stated otherwise, all compounds behaved as pure competitive antagonists and the curves were consistent with a one-site binding model. Most Hill coefficients (n_H) were not different from unity. Exceptions are given in grey italics in the corresponding tables.

4.1.2.1 Muscarinic M_{1-5} receptors

The affinity values (pK_i) of several reference drugs at muscarinic receptor subtypes are given in Table 4.2. The tested drugs spanned an affinity range of approximately three orders of magnitude and displayed different selectivity profiles. The obtained values are in good agreement with literature (Dörje et al., 1991; Böhme et al., 2003; K. Kreutzmann, personal communication). Examples of competition curves are shown in Fig. 4.5 displaying the curves of atropine, a highly potent, non-selective antagonist at M_{1-5} receptors.

Table 4.2 *pK_i values and Hill coefficients (in parentheses) of reference drugs at muscarinic subtypes determined in competition binding experiments with [^{3}H]NMS. Hill slopes significantly different from unity are given in grey italics.*

	M_1	M_2	M_3	M_4	M_5
Atropine	**8.89** ± 0.03	**8.59** ± 0.03	**8.84** ± 0.08	**8.94** ± 0.03	**8.63** ± 0.03
	(0.97 ± 0.01)	(1.00 ± 0.05)	(1.01 ± 0.07)	(0.96 ± 0.02)	(*0.93 ± 0.04*)
4-DAMP	**8.99** ± 0.05	**8.03** ± 0.05	**8.93** ± 0.02	**8.52** ± 0.02	**8.63** ± 0.03
	(0.90 ± 0.06)	(1.00 ± 0.05)	(1.02 ± 0.06)	(1.00 ± 0.02)	(0.96 ± 0.04)
(R)-Dimethindene	**5.73** ± 0.03	**5.91** ± 0.05	**5.47** ± 0.04	**5.41** ± 0.01	**5.57** ± 0.03
	(1.01 ± 0.03)	(1.01 ± 0.04)	(1.05 ± 0.07)	(0.99 ± 0.09)	(0.99 ± 0.03)
(S)-Dimethindene	**6.72** ± 0.05	**7.52** ± 0.05	**6.86** ± 0.01	**6.53** ± 0.05	**6.12** ± 0.03
	(*0.93 ± 0.02*)	(1.00 ± 0.04)	(1.02 ± 0.01)	(0.99 ± 0.03)	(1.02 ± 0.05)
Ipratropium	**8.93** ± 0.07	**9.14** ± 0.08	**9.30** ± 0.05	**9.18** ± 0.13	**8.71** ± 0.05
	(*0.92 ± 0.02*)	(1.02 ± 0.08)	(0.97 ± 0.02)	(0.98 ± 0.04)	(1.01 ± 0.10)
Pirenzepine	**7.93** ± 0.02	**6.37** ± 0.01	**6.70** ± 0.03	**7.26** ± 0.01	**6.86** ± 0.04
	(0.96 ± 0.03)	(1.01 ± 0.03)	(0.95 ± 0.03)	(*0.94 ± 0.02*)	(0.96 ± 0.11)

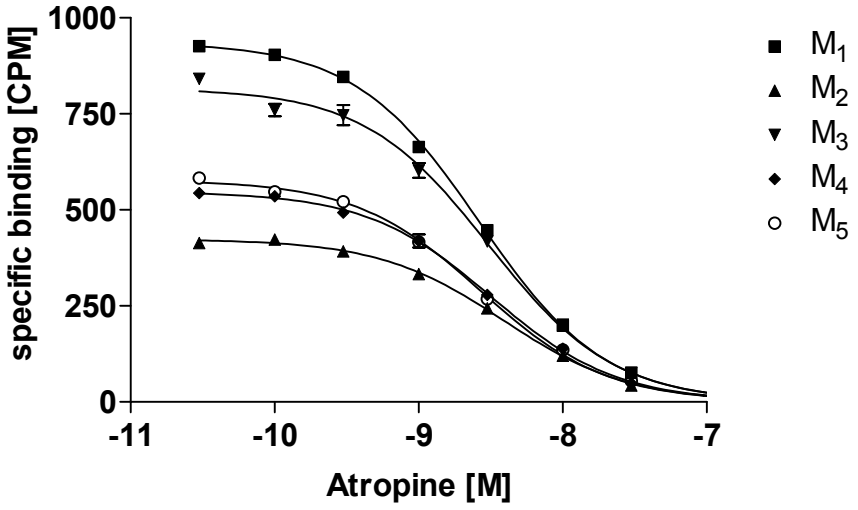


Fig. 4.5 *Competition curves of atropine at [^{3}H]NMS binding sites in membrane preparations from CHO-K1 cells stably expressing hM_{1-5} receptors.*

4.1.2.2 Serotonin 5-HT_{3A} receptor

The affinity values (pK_i) of several reference drugs at serotonin 5-HT_{3A} receptor are given in Table 4.3. The tested drugs spanned an affinity range of approximately five orders of magnitude. The obtained values are in good agreement with literature data for binding studies in tissue preparations of rat brain (Kilpatrick et al., 1987, 1989) and bovine brain (Miller et al., 1992). Good agreement can be seen to binding studies using mouse and rat neuroblastoma cells (Boeijinga et al., 1992; Lummis et al., 1990; Sharif et al., 1991). Examples of competition curves are shown in Fig. 4.6.

Table 4.3 *pK_i values and Hill coefficients (in parentheses) of reference drugs at serotonin h5-HT_{3A} receptors determined in competition binding experiments with [^{3}H]GR65630.*

	5-HT_{3A}
Alosetron	**9.69** ± 0.04 (1.06 ± 0.27)
Cocaine	**5.40** ± 0.09 (1.01 ± 0.12)
Dolasetron	**7.11** ± 0.06 (1.06 ± 0.02)
Granisetron	**9.10** ± 0.04 (1.05 ± 0.11)
McN-A-343	**4.96** ± 0.07 (1.01 ± 0.14)
MCP	**6.29** ± 0.08 (1.06 ± 0.09)
MDL72222	**8.30** ± 0.08 (1.10 ± 0.05)
Ondansetron	**8.83** ± 0.03 (1.09 ± 0.12)
Tropisetron	**8.87** ± 0.04 (1.08 ± 0.09)

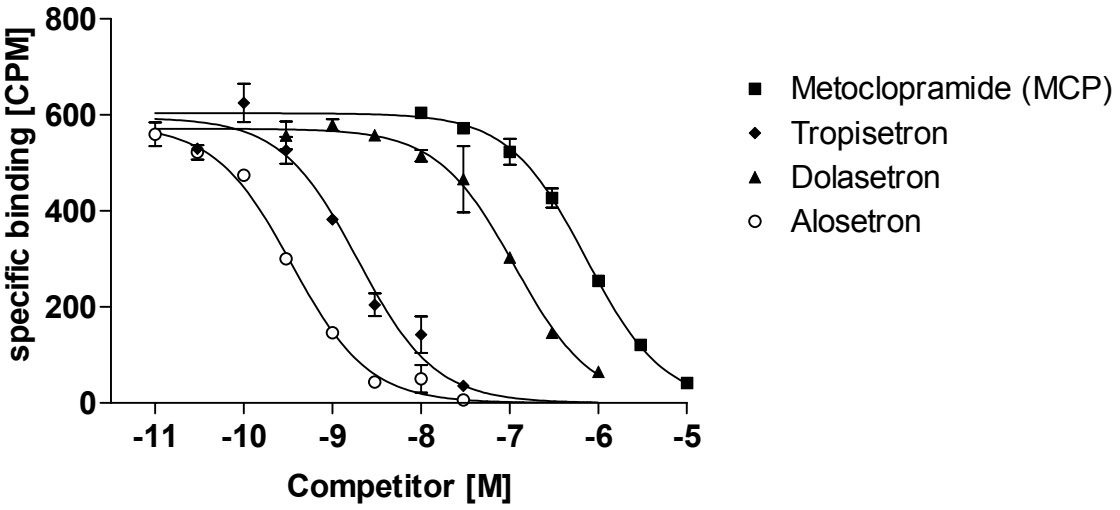


Fig. 4.6 *Competition curves of reference drugs at [^{3}H]GR65630 binding sites in membrane preparations from HEK293 cells stably expressing h5-HT$_{3A}$ receptors.*

4.1.2.3 Serotonin 5-HT$_{4(b)}$ receptor

Several reference drugs were tested with their affinity values spanning four orders of magnitude. Affinity data (pK_i values) are given in Table 4.4. Due to financial constraints these experiments were carried out only twice in duplicate. Only little data is available in literature using recombinant expression systems to measure 5-HT$_{4(b)}$ affinity. Most data was derived using guinea-pig brain preparations with the resulting affinities lying close to our data for McN-A-343 (Sagrada et al., 1994), GR113808 and RS39604 (Hegde and Eglen, 1996) and functional data in rat oesophagus for SB203186 (Hegde and Eglen, 1996) and RS23597-190 (Eglen et al., 1993b). One study using the 5-HT$_{4n}$ subtype stably expressed in HeLa cells revealed data close to ours concerning tropisetron (Vilaro et al., 2002). In general, our results were close to literature values with differences ranging from 0.3 - 0.5 log units. Competition curves of some reference drugs are given in Fig. 4.7.

Table 4.4 *pK_i values and Hill coefficients (in parentheses) of reference drugs at serotonin h5-$HT_{4(b)}$ receptors determined in competition binding experiments with [^{3}H]GR113808. Hill slopes significantly different from unity are given in grey italics.*

	5-HT_{4B}
GR113808	**9.73** ± 0.08 (0.97 ± 0.00)
RS39604	**8.83** ± 0.16 (1.00 ± 0.04)
RS23597-190	**7.28** ± 0.08 (*0.84 ± 0.01*)
SB203186	**8.39** ± 0.05 (1.00 ± 0.05)
Tropisetron	**6.41** ± 0.00 (0.97 ± 0.01)
McN-A-343	**5.73** ± 0.13 (1.03 ± 0.09)

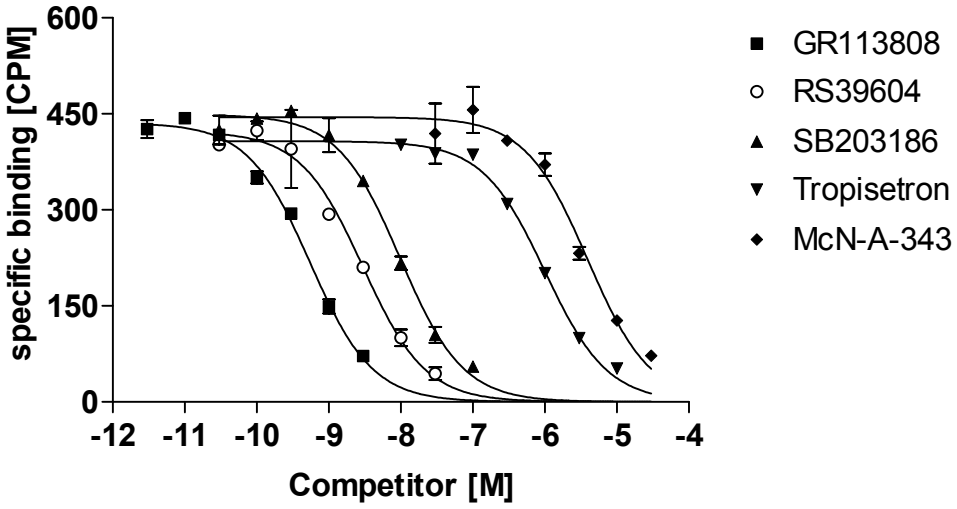


Fig. 4.7 *Competition curves of reference drugs at [^{3}H]GR113808 binding sites in membrane preparations from HEK293 cells stably expressing h5-$HT_{4(b)}$ receptors.*

4.1.2.4 Histamine H_1 receptor

Affinity data (pK_i values) of several reference drugs at H_1 receptors are summarized in Table 4.5. The tested drugs spanned an affinity range of approximately 1.5 orders of magnitude. The obtained values are in good agreement with literature values for binding studies at hH_1 receptors expressed in CHO-cells (Smit et al., 1996; Anthes et al., 2002). There is a striking consistence with binding data obtained in studies at H_1

receptor in guinea-pig brain tissue preparation (Ter Laak et al., 1993). Examples for competition curves are given in Fig. 4.8.

Table 4.5 *pK_i values and Hill coefficients (in parentheses) of reference drugs at histamine H_1 receptors determined in competition binding experiments with [^{3}H]mepyramine.*

	H_1
Astemizole	**8.72** ± 0.15 (1.19 ± 0.21)
Cetirizine	**7.28** ± 0.22 (1.07 ± 0.13)
Chlorpheniramine	**7.84** ± 0.01 (0.89 ± 0.10)
Diphenhydramine	**7.54** ± 0.12 (0.98 ± 0.16)

	H_1
Fexofenadine	**7.28** ± 0.01 (0.99 ± 0.10)
Mepyramine	**8.66** ± 0.07 (1.08 ± 0.08)
Terfenadine	**7.30** ± 0.07 (1.31 ± 0.30)
t-Triprolidine	**8.48** ± 0.10 (1.06 ± 0.08)

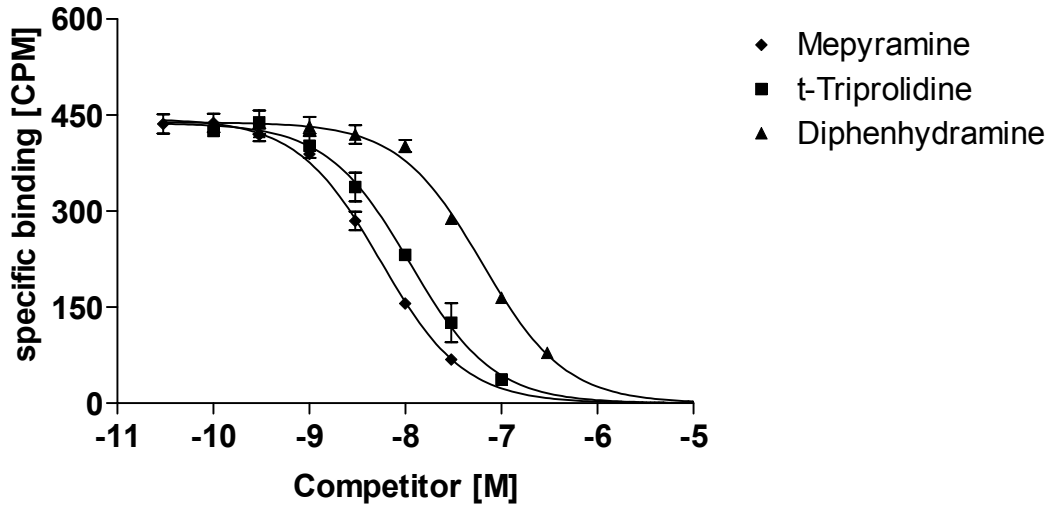


Fig. 4.8 *Competition curves of some reference drugs at [^{3}H]mepyramine binding sites in membrane preparations from CHO-K1 cells stably expressing hH_1 receptors.*

4.2 Analogues of ondansetron

Affinities values (pK_i) of the parent compound ondansetron (**1**) and derivatives **2A/B** - **6A/B** and **7** - **18** are given in Table 4.6. Reference values (pA_2) obtained in functional studies at 5-HT_3 receptors in guinea-pig ileum by the group of Prof. Dr. S. Elz, Regensburg, Germany, are shown, too. Ratios of affinities (h5-HT_{3A}/GPI) were calculated and added. One should notice that Hill coefficients of compounds **13** and **(+)14** were significantly different from unity. For these competition curves a two-site binding model was calculated in addition to a one-site binding model and the fits were compared with an F-Test. As in both cases the one-site binding model resulted in a better fit, pK_i values were derived from IC_{50} values although this is not permitted following the rules of Cheng-Prusoff in a strict sense. Those values should be handled with care. In later paragraphs pK_i values were derived in the same way unless stated otherwise when Hill slopes were significantly different from unity. Calculated pK_i values for derivatives of ondansetron (**1**) ranged from < 4.5 (**10**, **15**) up to 9.05 (**2A**), and h5-HT_{3A}/GPI ratios from < 0.03 (**15**) up to > 724 (**13**).

Table 4.6 *pK_i values and Hill coefficients (in parentheses) of ondansetron (**1**) and its analogues **2A/B** - **6A/B** and **7** - **18** determined in radioligand binding studies at h-5HT_{3A} receptors. Hill slopes significantly different from unity are given in grey italics. Reference values (pA_2) obtained in functional studies at 5-HT_3 receptors in guinea-pig ileum (GPI) and calculated ratios are shown in the last two columns.*

No.	h5-HT_{3A}	n_H	Ref. GPI	h5-HT_{3A} / GPI
1	**8.83** ± 0.03	(1.09 ± 0.12)	**7.00**	**68**
2A	**9.05** ± 0.09	(0.96 ± 0.09)	**7.37**	**48**
2B	**8.32** ± 0.03	(1.06 ± 0.08)	**6.13**	**155**
3A	**7.97** ± 0.11	(0.95 ± 0.04)	**6.30**	**47**
3B	**7.84** ± 0.08	(0.99 ± 0.05)	**5.60**	**174**
4A	**7.13** ± 0.02	(0.99 ± 0.04)	**5.80**	**21**
4B	**5.42** ± 0.10	(1.00 ± 0.06)	**< 4.30**	**> 13**
5A	**7.10** ± 0.11	(1.12 ± 0.13)	**6.75**	**2**
5B	**6.06** ± 0.05	(1.09 ± 0.23)	**5.85**	**2**

Table 4.6 (Continued)

No.	h5-HT_{3A}	n_H	Ref. GPI	h5-HT_{3A} / GPI
6A	**6.97** ± 0.06	(1.03 ± 0.11)	**7.15**	**0.7**
6B	**6.06** ± 0.11	(1.04 ± 0.18)	**6.16**	**0.8**
7	**8.20** ± 0.07	(1.02 ± 0.14)	**6.67**	**34**
8	**7.87** ± 0.05	(1.01 ± 0.09)	**5.91**	**91**
9	**7.43** ± 0.03	(1.04 ± 0.04)	**5.82**	**41**
10	**< 4.5**		**4.88**	**< 0.4**
11	**7.56** ± 0.04	(0.99 ± 0.07)	**5.92**	**44**
12	**7.75** ± 0.10	(0.93 ± 0.17)	**6.72**	**11**
13	**8.16** ± 0.16	*(1.06 ± 0.02)*	**< 5.3**	**> 724**
(+)-14	**5.00** ± 0.08	*(1.10 ± 0.03)*	**-**	**-**
(-)-14	**6.41** ± 0.06	(1.15 ± 0.16)	**-**	**-**
15	**< 4.5**		**6.08**	**< 0.03**
16	**6.74**± 0.05	(1.09 ± 0.21)	**6.08**	**5**
17	**6.77** ± 0.03	(1.14 ± 0.14)	**4.84**	**85**
18	**5.34** ± 0.10	(1.06 ± 0.10)	**5.25**	**1**

4.2.1 Analogues with substituents in position 4 and 5

Five pairs of regioisomers with substituents at position 4 (**2A** - **6A**) and 5 (**2B** - **6B**) of the imidazole ring were tested. pK_i values are given in Table 4.6. It can be clearly seen that substitution in position 4 ("**A**" series) is preferred versus position 5 ("**B**" series). Higher affinity values for the "**A**" series were found in binding and functional tests. Results are shown in Fig. 4.9. It is very interesting to notice, that the affinity values obtained at recombinant h5-HT_{3A} receptors were higher than that obtained at native gp5-HT_3 receptors in the case of **2A/B** - **4A/B** (up to 174-fold), almost identical in the case of **5A/B** (2-fold higher) or even smaller in the case of **6A/B** (factor 0.8 and 0.7). The highest affinities of all tested substances - apart from compound **1** (pK_i = 8.83) - were found for the regioisomers **2A** and **2B** (pK_i = 9.05 and 8.32, respectively), resulting in a 2-fold increase in affinity in comparison to compound **1** in the case of **2A**.

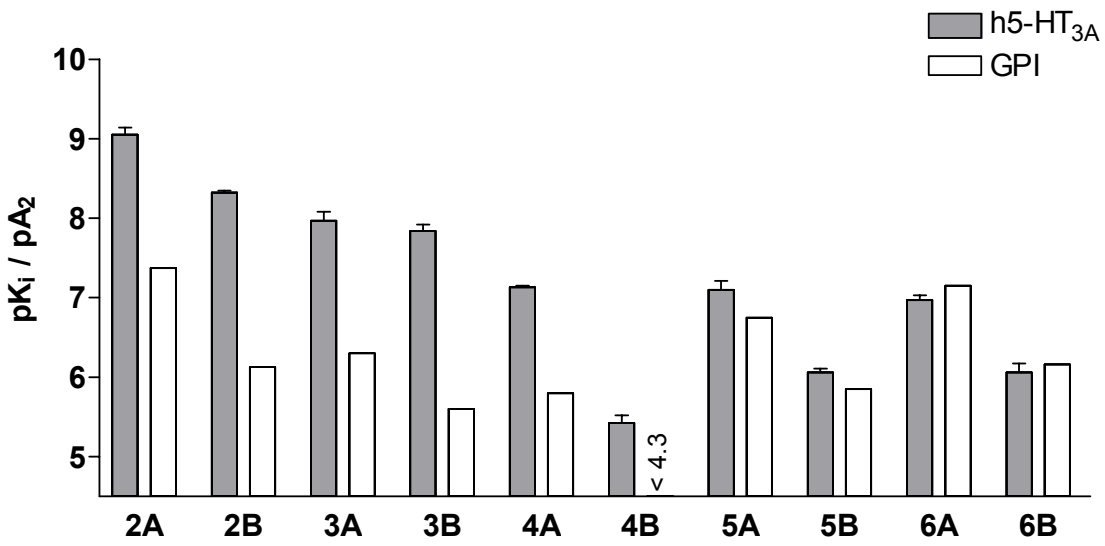


Fig. 4.9 *Affinity values of regioisomers with substituents at position 4 (**2A** - **6A**) or 5 (**2B** - **6B**) of the imidazole ring at h5-HT$_{3A}$ receptors (pK$_i$) and functional data obtained in guinea-pig ileum (pA$_2$).*

4.2.2 Analogues with modified substituents at position 2

Four compounds with modification at position 2 of the imidazole ring (**7** - **10**) were investigated and affinity values are shown in Table 4.6. The methyl group of **1** was exchanged with groups possessing a basic nitrogen or a phtalimide moeity. Fig. 4.10 (**A**) gives the affinity data for compounds **1** and **7** - **10**. All compounds in this group showed reduced affinities in comparison to the parent compound **1**. Changes in methylation pattern resulted only in minor affinity changes in compounds **7** and **8**. An aminomethyl substitution in compound **9** slightly decreased affinity. However, insertion of the nitrogen atom into a bulky substituent led to a dramatic loss of affinity in substance **10** reducing the pK$_i$ almost 3 orders of magnitude. This reduction of affinity was less pronounced in guinea-pig ileum. For compounds **1** and **7** - **9** pronounced higher affinities were found in binding studies than in functional studies (ratios ranging from 34 - 91), whereas the inverse was seen for compound **10** (ratio < 0.4).

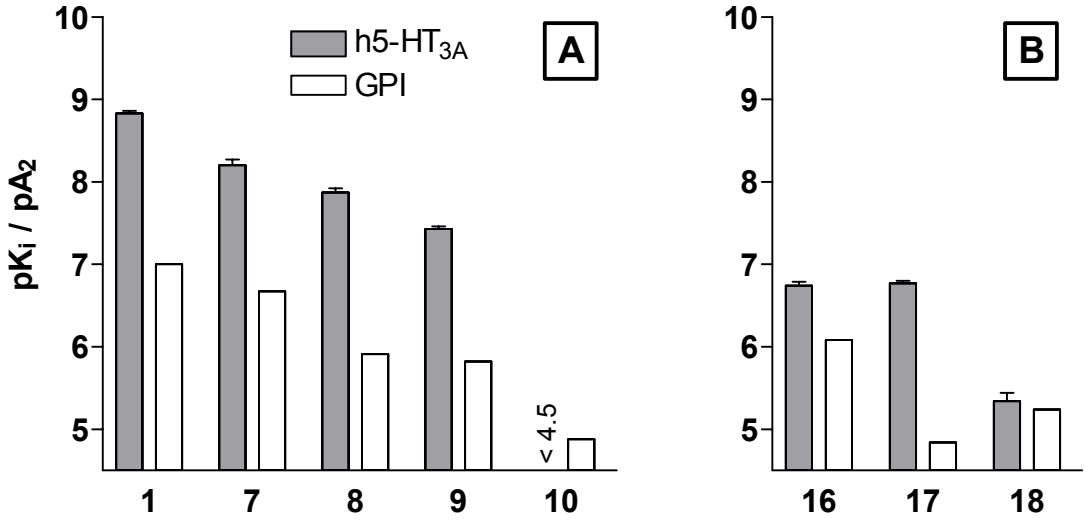


Fig. 4.10 *Affinity values of ondansetron (**1**) and its derivatives **7** - **10** with changed substituents at position 2 of the imidazole ring (**A**) and with changes in side chain and / or imidazole structure (**16** - **18**) (**B**) at h5-HT_{3A} receptors (pK_i) and functional data obtained in guinea-pig ileum (pA_2).*

4.2.3 Quaternized congeners, compounds with condensed ring and substances with a piperidine structure

N-Methylation of the nitrogen in position 3 of the imidazole ring of **1** led to the quaternary molecule **11** possessing comparable affinity to compound **12** with condensed aromatic ring. Affinity data is given in Table 4.6 and illustrated in Fig. 4.11. Exchanging the imidazole ring to a piperidine ring (**13**) resulted in high affinity in the low nanomolar range (pK_i = 8.16). However, for this compound the highest discrepancy between binding and functional studies was found. Affinity at h5-HT_{3A} was > 724-fold higher than at native gp5-HT_3 receptors. Introduction of a substituent in para-position of the piperidinyl moiety (**14** and **15**) led to a dramatic decrease in binding affinity. A considerable stereoselectivity ratio of 26 was seen for **(+)-14** and **(-)-14** even though absolute affinities were rather low (pK_i = 5.0 and 6.4, respectively). Introducing a bulky and sterically fixed substituent in compound **13**, led to a total loss of affinity at h5-HT_{3A} (4571-fold decrease) in compound **15**. The inverse effect was seen at native gp5-HT_3 receptors, where an at least 6-fold increase in affinity was found. Thus, for **15** the smallest ratio, recombinant vs. native 5-HT_3 receptors, was calculated (< 0.03).

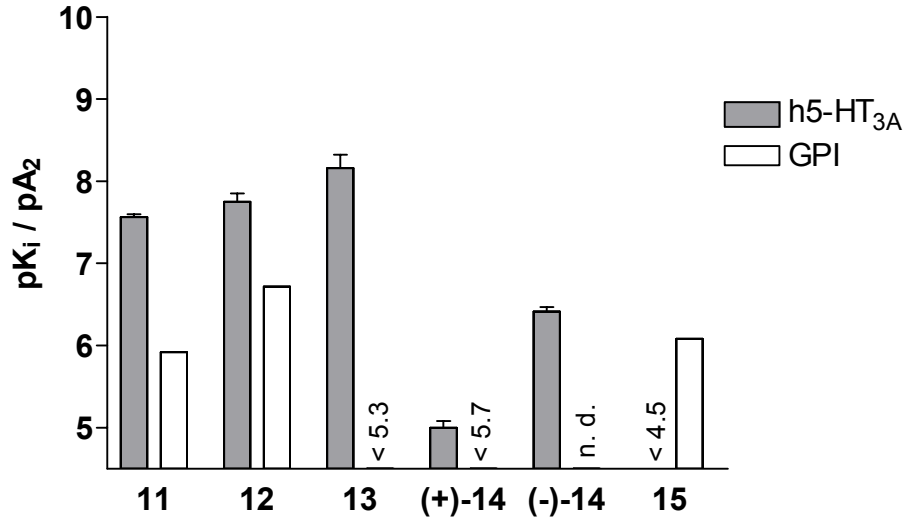


Fig. 4.11 *Affinity values of quaternary (**11**), condensed compounds (**12**) and derivatives with a piperidine moiety (**13** - **15**) at h5-HT_{3A} receptors (pK_i) and functional data obtained in guinea-pig ileum (pA_2). n. d. = not determined.*

4.2.4 Analogues with modifications in the side chain and / or imidazole ring

Three compounds (**16** - **18**) were tested with insertion of a basic nitrogen in the side chain. Data is given in Table 4.6, and illustrated in Fig. 4.10 (**B**). This modification led to a reduction in affinity of 2 orders of magnitude in the case of **16** and **17**, where the imidazole ring was conserved, and even more than 3 orders of magnitude in **18**, where this part was exchanged, with regard to the parent compound **1**. Compared to functional data, higher affinities were measured at h5-HT_{3A} receptors for compounds **16** and **17** (5-fold and 85-fold, respectively), whereas an almost identical affinity was found for substance **18**.

4.3 Analogues of metoclopramide

Affinities values (pK_i) of compound SDZ205-557 (**19**) and derivatives **20** - **30** are given in Table 4.7. Reference values obtained at 5-HT_4 receptors in guinea-pig ileum and rat oesophagus functional studies by the group of Prof. Dr. S. Elz, Regensburg, Germany, are shown, too. The obtained affinity values (pK_i) at h5-$HT_{4(b)}$ were in the range of 6.08 up to 8.43, and functional pA_2 values ranged from 5.7 to 8.5 in guinea-pig ileum and 5.8 to 8.4 in rat oesophagus, respectively.

Table 4.7 *pK_i values and Hill coefficients (in parentheses) of SDZ205-557 (**19**) and its analogues determined in radioligand binding studies at h5-$HT_{4(b)}$ receptors. Hill slopes significantly different from unity are given in grey italics. Reference values (pA_2) obtained in functional studies at 5-HT_4 receptors in guinea-pig ileum (GPI) and rat oesophagus (ROS) are shown in the last two columns.*

No.	h5-HT_{4B}	n_H	Ref. GPI	Ref. ROS
19	**7.83** ± 0.05	(0.96 ± 0.05)	**7.5**	**7.3**
(±)-20	**6.08** ± 0.09	(0.84 ± 0.09)	**5.7**	**5.8**
(±)-21	**6.75** ± 0.04	(1.00 ± 0.02)	**6.8**	**6.4**
22	**8.37** ± 0.02	(1.03 ± 0.02)	**8.5**	**8.4**
(+)-23	**7.27** ± 0.05	(1.02 ± 0.04)	**6.9**	**-**
(-)-23	**6.37** ± 0.11	(0.86 ± 0.11)	**6.6**	**-**
24	**7.39** ± 0.05	(0.99 ± 0.04)	**7.4**	**7.6**
25	**6.51** ± 0.05	(1.08 ± 0.05)	**7.1**	**7.4**
(±)-26	**8.43** ± 0.15	*(0.80 ± 0.03)*	**7.9**	**-**
27	**6.65** ± 0.05	(1.02 ± 0.07)	**6.8**	**-**
28	**6.87** ± 0.02	(1.04 ± 0.01)	**6.8**	**-**
29	**6.99** ± 0.03	(1.02 ± 0.04)	**7.3**	**-**
30	**8.28** ± 0.06	(0.94 ± 0.05)	**7.5**	**-**

4.3.1 Analogues with methylation in the side chain

Introducing a methyl substituent into the side chain next to basic nitrogen atom led to a loss in affinity for h5-$HT_{4(b)}$ receptors of approximately one order of magnitude for

(±)-21 in comparison to the unsubstituted parent compound **19** (pK_i values 6.75 and 7.83, respectively). Moving the methyl group to the carbon atom next to the ester oxygen, **(±)-21** → **(±)-20**, resulted in even lower affinity (pK_i = 6.08) (Table 4.7 and Fig. 4.12). The functional data obtained at native gp5-HT_4 and r5-HT_4 receptors were very similar to the binding affinities at recombinant h5-$HT_{4(b)}$ receptors.

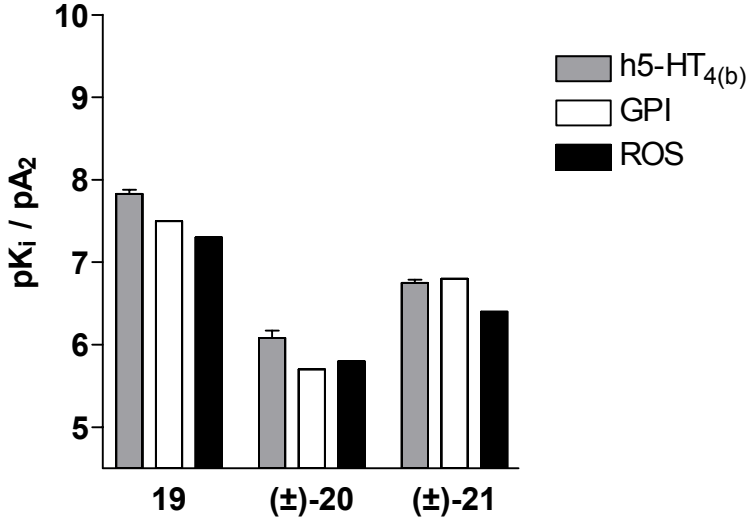


Fig. 4.12 *Affinity values of compounds with methyl groups in the side chain of **19** at h5-$HT_{4(b)}$ receptors (pK_i) and functional data obtained in guinea-pig ileum (GPI) and rat oesophagus (ROS) (pA_2).*

4.3.2 Analogues with a piperidine ring system

Insertion of the basic nitrogen of **19** in a piperidine ring system led to compounds **22** - **26**. Affinity data is given in Table 4.7, and illustrated in Fig. 4.13. For the piperidine congener **22** of the parent compound **19** a 4-fold increase in affinity was found (pK_i were 8.37 and 7.83, respectively). Compounds **(+)-23** and **(-)-23** were obtained by introduction of a methyl group in **22** next to the nitrogen atom. This resulted in decreased affinity (approximately one order of magnitude for the eutomer **(+)-23**). In contrast to functional studies in guinea-pig ileum, where almost no difference was seen for the enantiomers **(+)-23** and **(-)-23**, a stereoselectivity ratio of factor 8 was found in binding studies at h5-$HT_{4(b)}$ receptors. Elongation of the side chain length of compound **22** from 2 carbon atoms up to 3 (**24**) and 4 (**25**) carbon atoms led to clearly decreased affinities with increasing chain length. Introduction of a bulky aromatic substituent in 4 position of the piperidine ring in compound **22**, resulting in

(±)-26, was tolerated well without loss of affinity. This revealed this part of the molecule to be less critical than the side chain region with regard to binding affinity at 5-HT_4 receptors. With exception of compound **25**, there was no difference between functional and binding data. In the case of **25**, problems with solubility occurred when the substance was dissolved. Neither addition of methanol, DMSO nor hydrochloric acid led to complete dissolution. This may be the reason for the lower values found in our tests in comparison to functional tests.

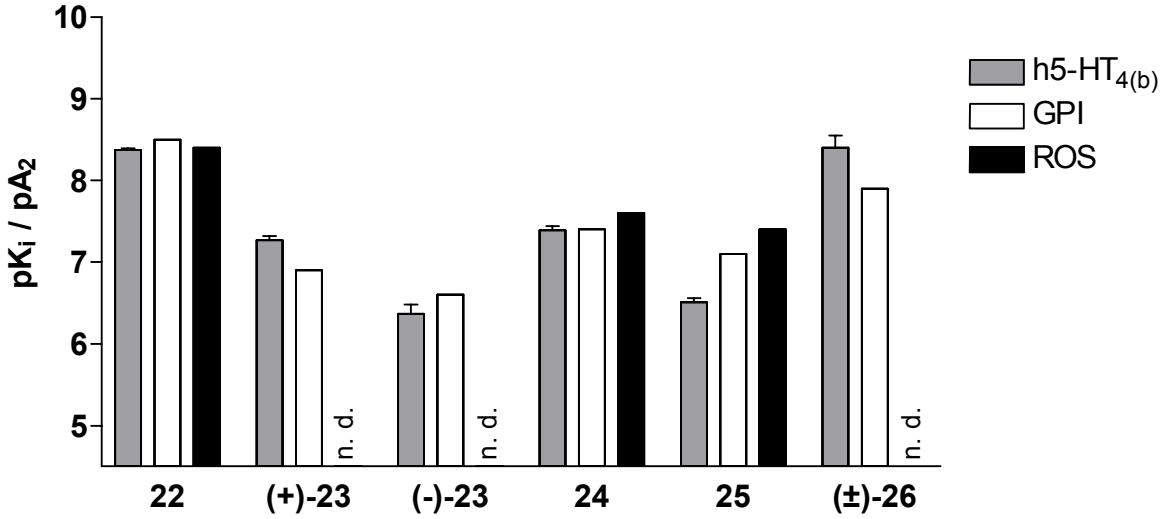


Fig. 4.13 *Affinity values of compounds with a piperidine ring system (**22** - **26**) and modifications in the side chain at h5-$HT_{4(b)}$ receptors (pK_i) and functional data obtained in guinea-pig ileum (GPI) and rat oesophagus (ROS) (pA_2). n. d. = not determined.*

4.3.3 Analogues with a piperazine ring system

Insertion of the basic nitrogen of **19** in a piperazine ring system resulted in compounds **27** - **30**. Affinity data is given in Table 4.7 and illustrated in Fig. 4.14. Compounds **27** - **29** have a benzyl-substituted piperazine ring with increasing side chain length (from 2 - 4 carbon atoms). Compared with the piperidine analogue **22** the introduction of a benzyl substituted piperazine ring (**27** - **29**) resulted in decreased binding affinities with only minor changes resulting in modification of chain length (pK_i values 8.37, 6.65, 6.87 and 6.99, respectively). Surprisingly, exchanging the benzyl substituent into a phenyl ring, **27** → **30**, restored binding affinity to a comparable level compared to the potent piperidine analogue **22**. For compounds **27**

- **29**, a good agreement was seen between functional and binding data, whereas a 6-fold discrepancy was found for **30**.

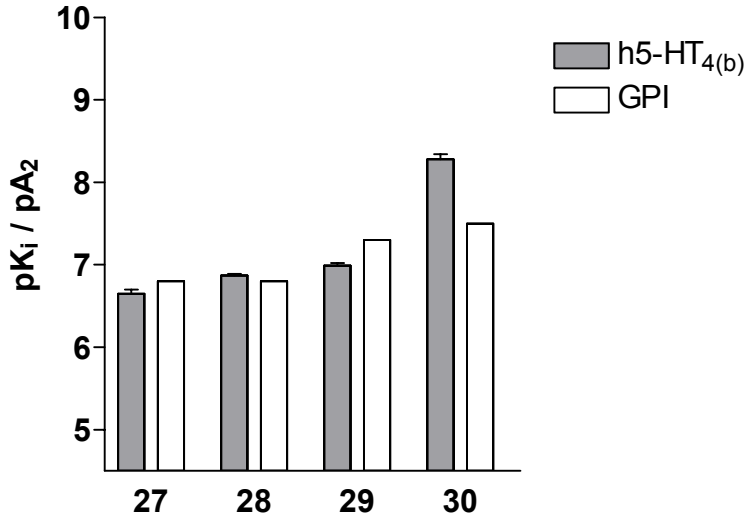


Fig. 4.14 *Affinity values of compounds with a piperazine ring system (**27** - **30**) at h5-HT$_{4(b)}$ receptors (pK$_i$) and functional data obtained in guinea-pig ileum (GPI) (pA$_2$).*

4.4 Analogues of McN-A-343

Affinity values (pK_i) of the parent compound McN-A-343 (**31**) and derivatives **32** - **42** at 5-HT_{3A}, 5-$HT_{4(b)}$ and H_1 receptors are listed in Table 4.8. Reference values obtained in previous binding studies at muscarinic M_1 receptors in our laboratories by Dr. C. Keim (2000) are added for the most interesting compounds discussed later on.

Table 4.8 *pK_i values and Hill coefficients (in parentheses) of McN-A-343 (**31**) and its analogues (**32** - **42**) determined in radioligand binding studies at h5-HT_{3A}, h5-$HT_{4(b)}$ and hH_1 receptors. Hill slopes significantly different from unity are given in grey italics. Reference values (pK_i) obtained in binding studies at M_1 receptors are shown in the last column.*

No.	5-HT_{3A} [a]	5-$HT_{4(b)}$	H_1	Ref. M_1 [b]
31	**4.96** ± 0.07 (1.01 ± 0.14)	**5.73** ± 0.13 (1.03 ± 0.09)	**< 4.5**	
32	**16%**	**4.70** ± 0.06 (1.02 ± 0.03)	**< 4.5**	
32$^+$	**10%**	**4.75** ± 0.05 (1.11 ± 0.07)	**< 4.5**	
(R)-33	**< 4.5**	**4.84** ± 0.04 (1.23 ± 0.15)	**5.43** ± 0.12 (1.23 ± 0.42)	**5.47**
(S)-33	**6%**	**4.69** ± 0.05 (1.75 ± 0.56)	**4.93** ± 0.06 (0.99 ± 0.26)	**7.40**
(R)-33$^+$	**5.74** ± 0.19 (1.15 ± 0.19)	**4.75** ± 0.02 (0.96 ± 0.03)	**4.82** ± 0.11 (0.88 ± 0.11)	**5.90**
(S)-33$^+$	**10%**	**4.60** ± 0.06 *(1.17 ± 0.04)*	**< 4.5**	**7.67**
(±)-34	**< 4.5**	**-**	**4.75** ± 0.09 (0.86 ± 0.30)	
(±)-34$^+$	**5.58** ± 0.02 *(1.44 ± 0.17)*	**-**	**< 4.5**	
(R)-35	**15%**	**5.06** ± 0.08 (0.99 ± 0.07)	**5.09** ± 0.03 (0.86 ± 0.15)	
(S)-35	**13%**	**4.85** ± 0.05 (1.09 ± 0.09)	**4.82** ± 0.06 (0.97 ± 0.03)	

Table 4.8 (continued)

No.	5-HT$_{3A}$ [a)]	5-HT$_{4(b)}$	H_1	Ref. M_1 [b)]
(R)-35$^+$	**14%**	**5.26** ± 0.05 (1.01 ± 0.04)	**< 4.5**	
(S)-35$^+$	**4%**	**5.09** ± 0.09 (0.96 ± 0.07)	**< 4.5**	
(R)-36	**21%**	**5.20** ± 0.07 (1.23 ± 0.34)	**6.71** ± 0.06 (0.84 ± 0.10)	**7.20**
(S)-36	**28%**	**5.23** ± 0.09 (1.15 ± 0.08)	**5.78** ± 0.09 (1.02 ± 0.08)	**9.40**
(R)-36$^+$	**24%**	**5.23** ± 0.03 (1.03 ± 0.07)	**6.26** ± 0.06 (1.00 ± 0.11)	**6.66**
(S)-36$^+$	**31%**	**5.00** ± 0.08 (0.98 ± 0.03)	**4.71** ± 0.02 (0.92 ± 0.15)	**8.80**
(R)-37	**4.65** ± 0.11 (0.87 ± 0.12)	-	**6.72** ± 0.08 (0.92 ± 0.20)	**6.18**
(S)-37	**18%**	-	**5.68** ± 0.10 (0.98 ± 0.00)	**9.40**
(R)-37$^+$	**18%**	-	**6.04** ± 0.02 *(0.83 ± 0.04)*	**6.87**
(S)-37$^+$	**32%**	-	**< 4.5**	**8.76**
(±)-38	**24%**	-	**6.98** ± 0.10 (0.86 ± 0.18)	
(±)-38$^+$	**18%**	-	**6.30** ± 0.11 (1.09 ± 0.16)	
(±)-39	**5.25** ± 0.09 *(0.63 ± 0.19)*	-	**6.27** ± 0.09 (1.37 ± 0.40)	
(±)-39$^+$	**5.22** ± 0.02 (1.03 ± 0.09)	-	**5.12** ± 0.15 *(0.68 ± 0.16)*	
(±)-40	**4.90** ± 0.09 *(0.74 ± 0.09)*	-	**6.12** ± 0.05 (1.00 ± 0.02)	
(±)-40$^+$	**4.81** ± 0.04 (1.11 ± 0.23)	-	**5.86** ± 0.04 (1.01 ± 0.04)	
(±)-41	**5.45** ± 0.05 (0.96 ± 0.05)	-	**6.74** ± 0.05 (1.05 ± 0.14)	

Table 4.8 (continued)

No.	5-HT_{3A} [a)]	5-$HT_{4(b)}$	H_1	Ref. M_1 [b)]
(±)-41$^+$	**24%**	-	**6.07** ± 0.11 (1.06 ± 0.26)	
(±)-42	**5.08** ± 0.02 (1.03 ± 0.02)	-	**6.58** ± 0.06 (1.23 ± 0.20)	
(±)-42$^+$	**32%**	-	**5.71** ± 0.08 (0.98 ± 0.05)	

[a)] Values given in percent were obtained in one point screening experiments using a concentration of 1 x 10^{-5} M of cold competitor. Tests were carried out twice in duplicate. Values given are the means of inhibition of specific binding.

[b)] Reference values of selected compounds at M_1 receptors were measured in previous tests in our laboratory by Dr. C. Keim (2000) in binding experiments.

4.4.1 Studies at 5-HT_{3A} receptors

Binding data for compounds tested at this subtype are shown in Table 4.8. All values given in percent equal a pK_i value in the range of 4.5 - 5.0 or below. The affinity value determined for **31** was in good agreement with that reported in previous work (Sagrada et al., 1994). pK_i values of tested derivatives culminated at 5.74 for compound **(R)-33$^+$** displaying a 6-fold increase in comparison to the parent compound. This was disappointing as all structural modifications led only to minor changes in affinity. For the quaternary compounds **(R/S)-33$^+$** stereoselectivity was found. The (R)-configurated enantiomer displayed pronounced higher affinity than the (S)-configurated compound (pK_i = 5.74 and < 4.5, respectively). It is astonishing that this fact was not found for the tertiary congeners **(R/S)-33** which both did not display significant affinities. No preference for tertiary or quaternary compounds concerning binding affinity at this subtype was observed.

4.4.2 Studies at 5-$HT_{4(b)}$ receptors

Due to financial constraints only a comparably small number of compounds was tested at this subtype. Data are shown in Table 4.8. Binding data for the parent compound **31** were in good agreement with literature (Sagrada et al., 1994). Experiments with the tested 14 congeners revealed decreased affinities, ranging

from pK_i = 4.60 to 5.26. No preference for tertiary or quaternary compounds could be detected. A very small stereoselectivity effect was found in favour of the (R)-configurated compounds in the cases of **(R/S)-33**, **(R/S)33$^+$**, **(R/S)-35**, **(R/S)-35$^+$** and **(R/S)-36$^+$** (Fig. 4.15). All modifications introduced in **31** led to a decrease in affinity of at least factor 3 in the case of the most potent derivative **(R)-35$^+$** (pK_i = 5.26). Changing the substitution pattern in the aromatic aniline ring into a 4-fluoro substituent (**32**, **32$^+$**) led to reduction in affinity by one order of magnitude. A phenyl substituent attached to the chain (**36**, **36$^+$**) provided comparable affinities to the methyl substituted compounds (**35**, **35$^+$**). Therefore, it can be concluded that this is a less critical point in the molecule compared to the substitution pattern of the aromatic aniline phenyl ring.

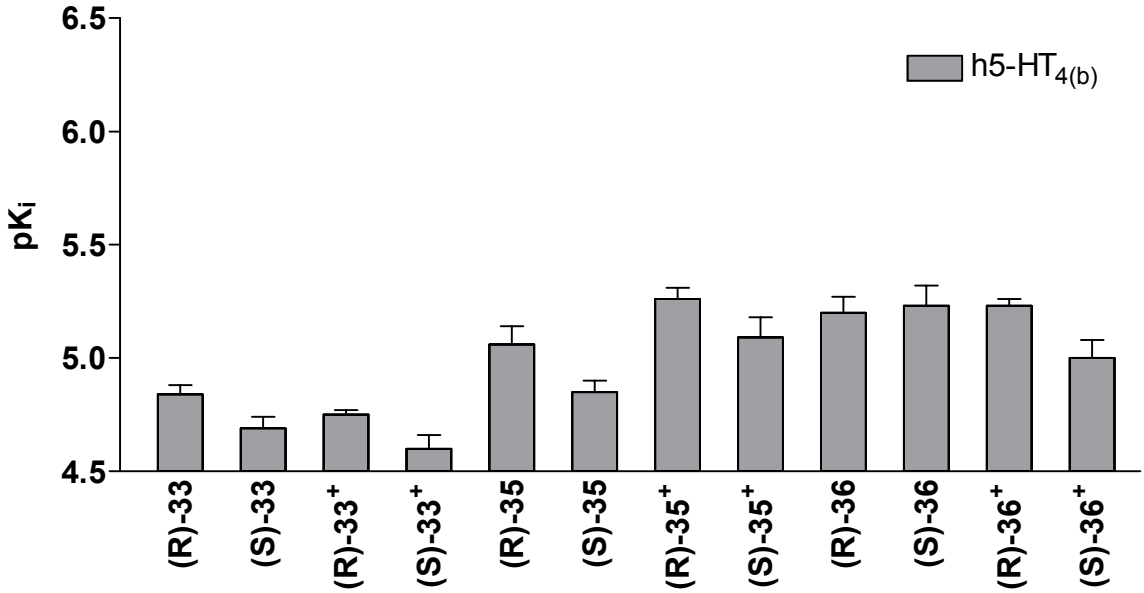


Fig. 4.15 *Affinity values (pK_i) of chiral compounds tested at h5-$HT_{4(b)}$.*

4.4.3 Studies at H_1 receptors

Since considerable efforts were made in our laboratory concerning the affinity of derivatives of McN-A-343 (**31**) at muscarinic receptor subtypes (see also chapter 2.4), analogues listed in Table 4.8 (**32** - **42**) were tested at the closely related histamine H_1 receptor. Obtained affinities were spanning a range from pK_i < 4.5 - 6.98.

4.4.3.1 Compounds with acyclic amino moiety

The achiral 4-fluoro-substituted congeners **32**, **32**$^{+}$ as well as McN-A-343 (**31**) had no detectable affinity for this subtype in binding experiments. Chiral compounds **(R/S)-33** and **(R/S)-33**$^{+}$ with attached C1-phenyl substituent displayed only low affinities at the micromolar range. **(±)-34** with an additional o-phenyl substituent had a low affinity, too. Taken together, derivatives with an acyclic amine moiety showed only low affinities with the highest affinity measured for **(R)-33** (pK_i = 5.4) being at least 9-fold more potent than the parent compound **31**.

4.4.3.2 Derivatives with a pyrrolidine ring system

Analogues with 4-fluoro-substituted aromatic aniline phenyl rings and different C1-substituents attached to the chain starting from a simple methyl **(R/S)-35**, **(R/S)-35**$^{+}$ via phenyl **(R/S)-36**, **(R/S)-36**$^{+}$ and 4-F-phenyl **(±)-38**, **(±)-38**$^{+}$ to a bulky 1-naphthyl substituent **(±)-39**, **(±)-39**$^{+}$ were tested. In addition, the congeners of **36** without substituent in the aromatic ring **37**, **37**$^{+}$ were examined. Data are given in Table 4.8 and are illustrated in Fig. 4.16. It could be seen that affinities increased with increasing size of the substituent starting at **(R)-35** to **(R)-36** reaching the highest affinity value for **(±)-38** (pK_i = 5.09, 6.71, 6.98, respectively). Exchanging the methyl group into a phenyl ring (**35** → **36**) increased affinity 42-fold. Only a small (2-fold) further gain in affinity was reached with a 4-F-phenyl substituent (**36** → **38**). Changing the phenyl ring into a bigger 1-naphthyl substituent (**38** → **39**) decreased affinity 5-fold. **(±)-38** showed the highest H_1 affinity of all tested compounds within this series and was > 302-fold more potent than the parent compound **31**.

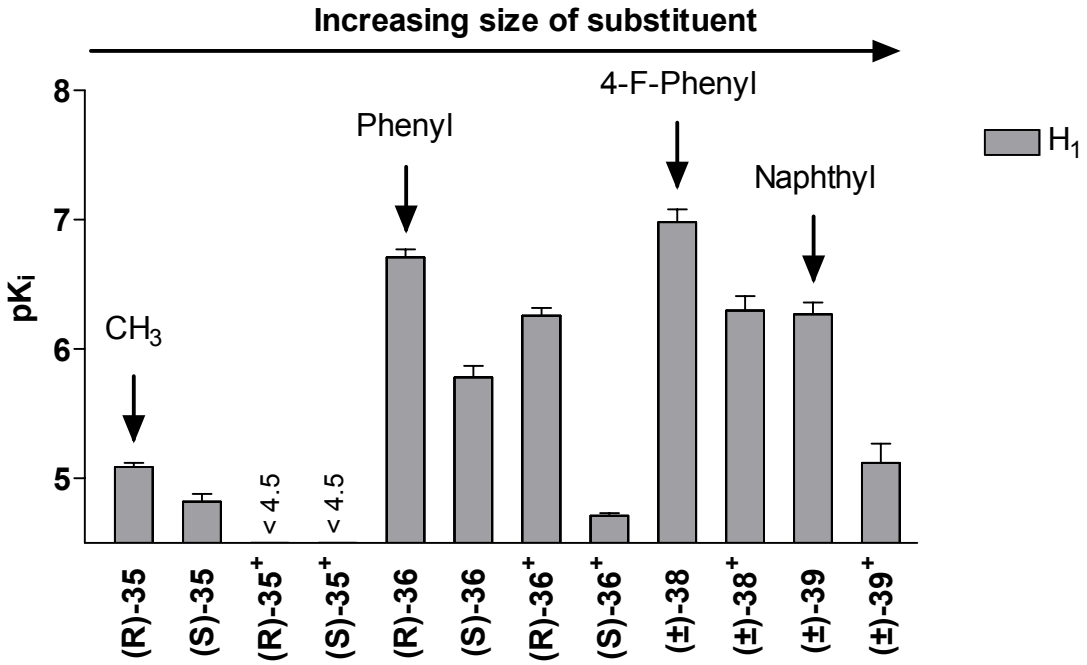


Fig. 4.16 *Affinity values (pK_i) of compounds with a pyrrolidine ring system and an C1-substituent in the chain (**35**, **36**, **38** and **39**) at H_1 receptor. The arrows mark the tertiary eutomers or racemates.*

In Fig. 4.17 the closely related compounds **36** and **37**, differing only in a fluorine atom at the aromatic ring, were compared. This F → H exchange was bioisoster as binding data were almost identical, with non of the analogues displaying a difference in affinity above 2-fold at H_1 receptor.

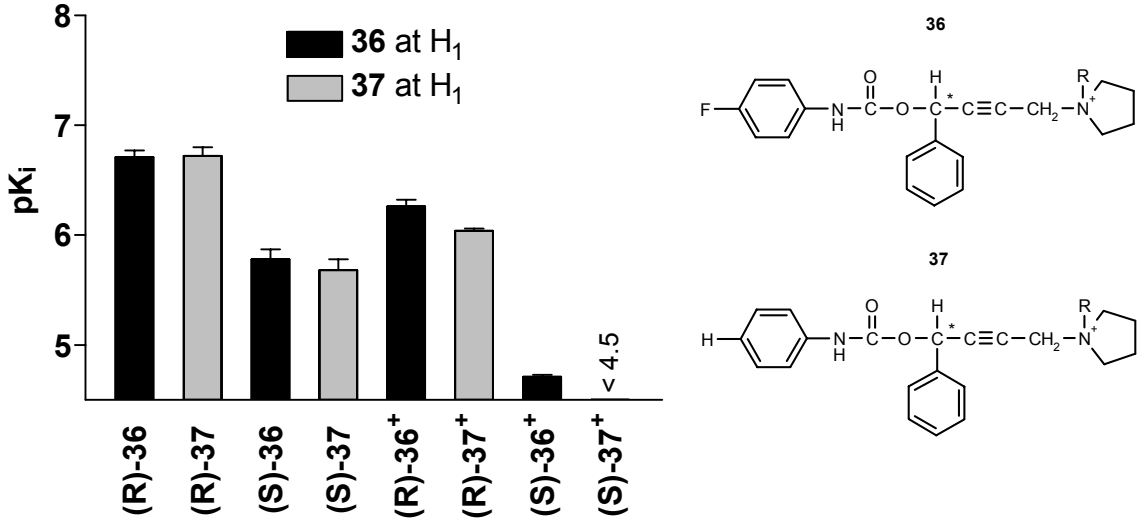


Fig. 4.17 *Affinity values (pK_i) of tertiary and quaternary enantiomers of **36** and **37** at H_1 receptors.*

Compounds possessing a pyrrolidine ring instead of an acyclic amine moiety showed much higher affinities. This could be seen especially well in the analogues **33** and **37** differing only in that part of the molecule. Data in Fig. 4.18 illustrate this pronounced, almost 20-fold increase in affinity in the case of the tertiary (R)-configurated compounds.

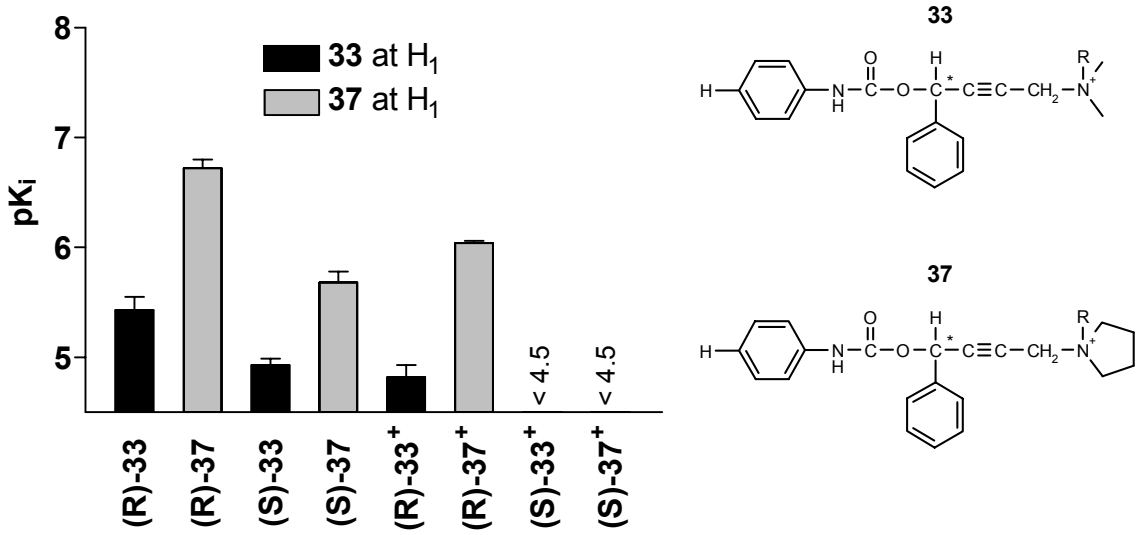


Fig. 4.18 *Affinity values (pK_i) of tertiary and quaternary enantiomers of **33** and **37** at H_1 receptors.*

4.4.3.3 Compounds with exchange of the aniline or carbamate moiety

Introducing a 1-naphthyl substituent (**40**) resulted in 4-fold lower affinity compared to the phenyl analogue eutomer **(R)-37** (pK_i = 6.12 and 6.72, respectively). Interestingly, exchange of the phenyl ring into a diphenyl-methyl moiety (**37** → **41**) was tolerated without decrease in affinity (pK_i = 6.72 and 6.74, respectively). Exchange of the carbamate structure into a carboxylic ester function (**36** → **42**) was tolerated. Data are illustrated in Fig. 4.19.

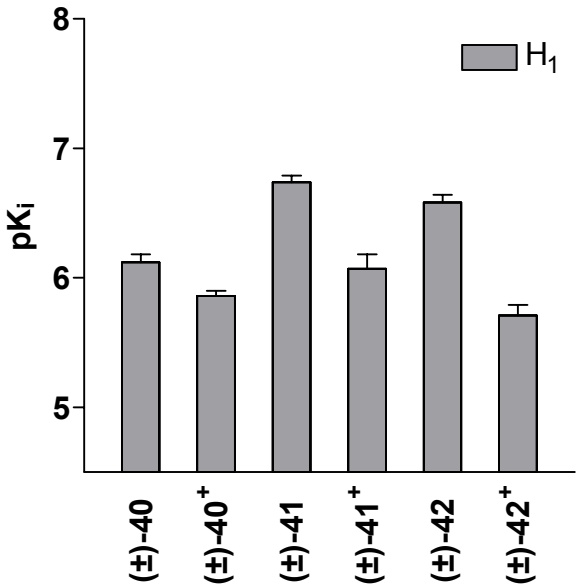


Fig. 4.19 *Affinity values (pK_i) of tertiary and quaternary compounds with exchanged aniline (**40** and **41**) or carbamate moiety (**42**) at H_1 receptors.*

4.5 Analogues of glycopyrronium

Affinity values (pK_i) of parent compound **43D** and analogues **44** - **56** at muscarinic hM_{1-5} receptors are given in Table 4.9, being in the range of 5.53 - 10.70.

Table 4.9 *pK_i values and Hill coefficients (in parentheses) of **43D** and analogues **44 - 56** determined in radioligand binding studies at hM_{1-5} receptors. Hill slopes significantly different from unity are given in grey italics.*

No.	M_1	M_2	M_3	M_4	M_5
43D[a)]	**9.94**	**9.70**	**9.95**	**9.90**	**9.35**
Dia-44D	**9.60** ± 0.15	**9.48** ± 0.03	**9.69** ± 0.07	**9.63** ± 0.07	**9.23** ± 0.01
	(1.02 ± 0.06)	(0.95 ± 0.06)	(1.00 ± 0.09)	(0.98 ± 0.05)	(0.99 ± 0.04)
Dia-45D	**9.80** ± 0.13	**9.82** ± 0.10	**9.78** ± 0.06	**9.79** ± 0.05	**9.36** ± 0.04
	(1.08 ± 0.05)	(0.91 ± 0.10)	(1.04 ± 0.04)	(0.96 ± 0.15)	(0.94 ± 0.05)
Dia-46D	**9.53** ± 0.17	**9.10** ± 0.06	**9.54** ± 0.05	**9.50** ± 0.05	**8.97** ± 0.07
	(1.01 ± 0.04)	(0.94 ± 0.04)	*(1.15 ± 0.06)*	(1.00 ± 0.02)	(1.10 ± 0.05)
Dia-47D	**9.59** ± 0.12	**9.27** ± 0.23	**9.53** ± 0.07	**9.61** ± 0.07	**9.27** ± 0.01
	(1.28 ± 0.23)	*(1.26 ± 0.07)*	*(1.30 ± 0.04)*	(1.21 ± 0.13)	(1.26 ± 0.20)
Dia-48D	**9.74** ± 0.16	**9.95** ± 0.17	**9.61** ± 0.12	**9.84** ± 0.13	**9.44** ± 0.07
	(0.92 ± 0.06)	(1.00 ± 0.08)	*(0.93 ± 0.02)*	(0.89 ± 0.06)	(1.01 ± 0.07)
49A	**7.88** ± 0.04	**7.61** ± 0.11	**6.85** ± 0.14	**7.55** ± 0.14	**7.69** ± 0.08
	(0.63 ± 0.12)	(0.88 ± 0.05)	(0.76 ± 0.20)	(0.76 ± 0.43)	*(0.78 ± 0.10)*
49B	**8.27** ± 0.07	**7.60** ± 0.12	**7.63** ± 0.11	**7.56** ± 0.07	**7.85** ± 0.08
	(0.93 ± 0.23)	(0.85 ± 0.09)	(0.92 ± 0.05)	(1.06 ± 0.18)	(1.02 ± 0.19)
49C	**9.76** ± 0.10	**9.18** ± 0.09	**9.44** ± 0.06	**9.59** ± 0.06	**8.83** ± 0.09
	(1.36 ± 0.15)	(1.17 ± 0.09)	(1.31 ± 0.30)	(1.50 ± 0.36)	(1.31 ± 0.23)
49D[a)]	**9.91**	**9.98**	**9.96**	**10.19**	**9.45**
50D	**9.96** ± 0.09	**10.17** ± 0.07	**9.87** ± 0.07	**9.94** ± 0.11	**9.52** ± 0.10
	(1.08 ± 0.05)	(1.03 ± 0.04)	(1.28 ± 0.15)	(1.33 ± 0.31)	(1.36 ± 0.28)
51D	**10.33** ± 0.19	**10.70** ± 0.06	**10.17** ± 0.04	**10.39** ± 0.16	**9.61** ± 0.07
	(0.90 ± 0.10)	(0.99 ± 0.09)	*(0.68 ± 0.10)*	(0.90 ± 0.08)	(1.02 ± 0.09)

Table 4.9 (continued)

No.	M_1	M_2	M_3	M_4	M_5
52	**7.86** ± 0.17	**7.89** ± 0.05	**8.05** ± 0.08	**7.95** ± 0.07	**7.58** ± 0.08
	(1.12 ± 0.09)	(1.24 ± 0.13)	*(1.13 ± 0.05)*	(1.07 ± 0.06)	*(1.16 ± 0.07)*
Dia-53	**6.36** ± 0.15	**6.17** ± 0.20	**6.41** ± 0.02	**6.44** ± 0.02	**6.33** ± 0.03
	(1.03 ± 0.04)	(0.94 ± 0.06)	(1.08 ± 0.09)	(1.06 ± 0.08)	(1.05 ± 0.08)
(R)-54	**6.01** ± 0.12	**5.99** ± 0.18	**5.88** ± 0.10	**6.01** ± 0.06	**5.53** ± 0.08
	(0.89 ± 0.20)	(1.01 ± 0.16)	(0.97 ± 0.01)	(0.95 ± 0.04)	(0.95 ± 0.10)
(R)-55	**9.47** ± 0.15	**9.94** ± 0.10	**9.24** ± 0.09	**9.33** ± 0.05	**8.79** ± 0.07
	(1.01 ± 0.04)	(1.10 ± 0.06)	(0.96 ± 0.06)	(1.02 ± 0.05)	(1.05 ± 0.13)
(R)-56	**10.11** ± 0.10	**10.19** ± 0.04	**10.03** ± 0.07	**9.82** ± 0.08	**9.66** ± 0.02
	(0.97 ± 0.17)	(0.82 ± 0.08)	(0.84 ± 0.19)	(0.95 ± 0.16)	(0.84 ± 0.28)

[a] *Values were measured by K. Kreutzmann (personal communication).*

4.5.1 Analogues with a pyrrolidine ring system

The exchange of a methyl group at the quaternary nitrogen of **43D** resulted in the racemic compounds **Dia-44D** - **Dia-48D**. Neither introducing unsaturated moieties **Dia-44D**, **Dia-45D** nor saturated **Dia-46D** or aromatic substituents **Dia-47D**, **Dia-48D** at this position resulted in a significant change of binding affinities at muscarinic receptor subtypes. All compounds showed pK_i values between 9.0 - 10.0 and displayed no selectivity for any subtype. Data are given in Table 4.9 and illustrated in Fig. 4.20 for **Dia-44D** - **Dia-46D** and Fig. 4.21 (**A**) for **Dia-47D**, **Dia-48D**.

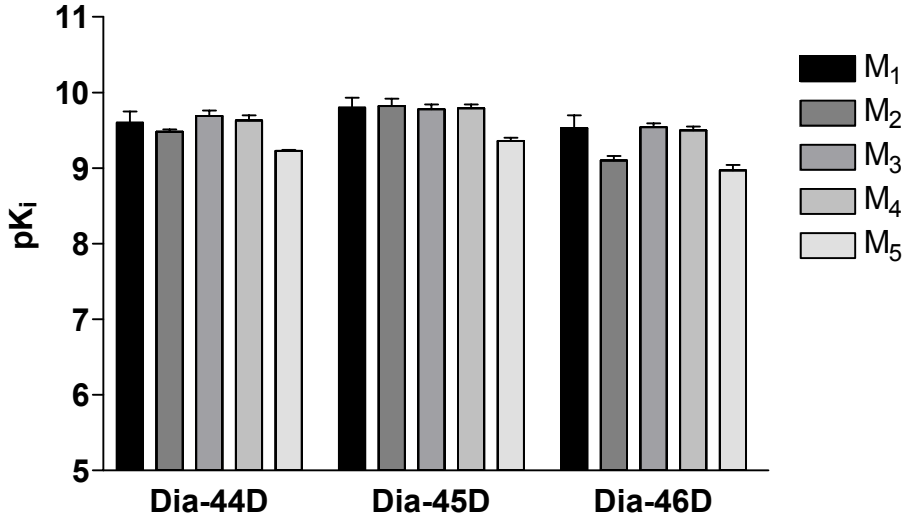


Fig. 4.20 *Affinity values (pK_i) at $M_{1\text{-}5}$ receptors of racemic compounds **Dia-44D** - **Dia-46D** containing a pyrrolidine ring and a chiral quaternary nitrogen.*

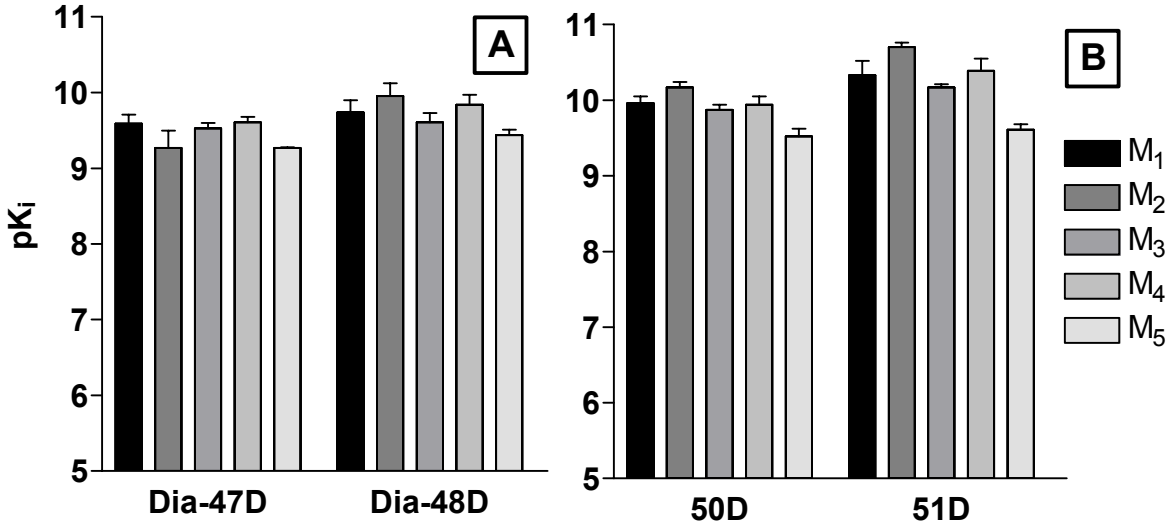


Fig. 4.21 *Affinity values (pK_i) at $M_{1\text{-}5}$ receptors of racemic compounds **Dia-47D** and **Dia-48D** containing a pyrrolidine ring and an aromatic substituent at the quaternary nitrogen (**A**) and their chinuclidine congeners **50D** and **51D** (**B**).*

4.5.2 Compounds with a chinuclidine ring system

Exchange of the pyrrolidine ring of the parent compound **43** into a chinuclidine ring led to the 4 stereoisomers of **49** (**49A-D**). Binding data are given in Table 4.9 and are illustrated in Fig. 4.22. It could be seen that none of the compounds had significant

subtype selectivity. The (2'S)-configurated compounds **49A** and **49B** had an approximately 100 - 300-fold decreased affinity in comparison to the (3R, 2'R)-configurated compound **49D** (K. Kreutzmann, personal communication). Only minor losses in affinities were found for the (3S, 2'R)-configurated isomer **49C** with a maximum 6-fold decrease in the case of the M_2 subtype.

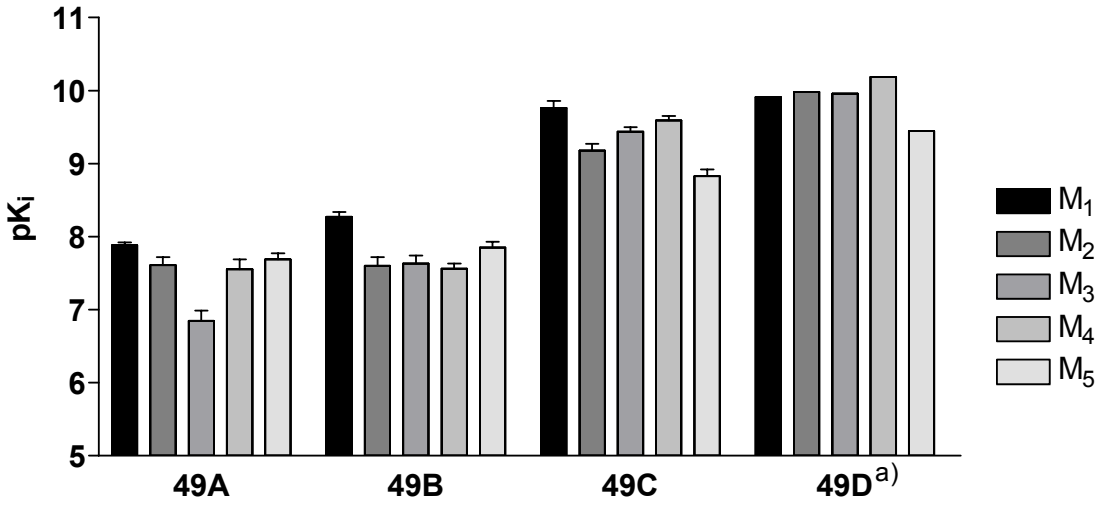


Fig. 4.22 *Affinity values (pK_i) of **49A-D** at M_{1-5} receptors. [a)] Values were measured by K. Kreutzmann (personal communication).*

Further on, compounds **50D** and **51D** were tested, both having an aromatic substituent at the quaternary nitrogen of the chinuclidine ring system. Graphs are shown in Fig. 4.21 (**B**) vis-à-vis its pyrrolidine congeners **Dia-47D** and **Dia-48D** (Fig. 4.21 (**A**)). The highest affinity value in the whole series was measured at the M_2 subtype for **51D** (pK_i = 10.70). **51D** showed slightly higher affinities than **50D** at M_{1-4} receptors. **50D** had affinities comparable to the parent compound **43D**, whereas **51D** was even more potent at the $M_{1,2,4}$ subtypes (up to 10-fold in case of the M_2 subtype).

4.5.3 Dimerised molecules and synthesis precursors

Compound **52** consists of two molecules **43D** linked with a aliphatic spacer. In **52** the quaternary nitrogen atoms are additional chiral centres, leading to a total of six centres of chirality in this molecule. In our tests a mixture of the resulting stereoisomers was used. Dimerisation led to an approximately 100-fold decrease in affinity at all subtypes. Data are given in Table 4.9 and are illustrated in Fig. 4.23.

Further on, the affinity data of precursors **Dia-53** and **(R)-54** are shown. Affinities for these precursors were more than three orders of magnitude lower in comparison to the parent compound **43D**.

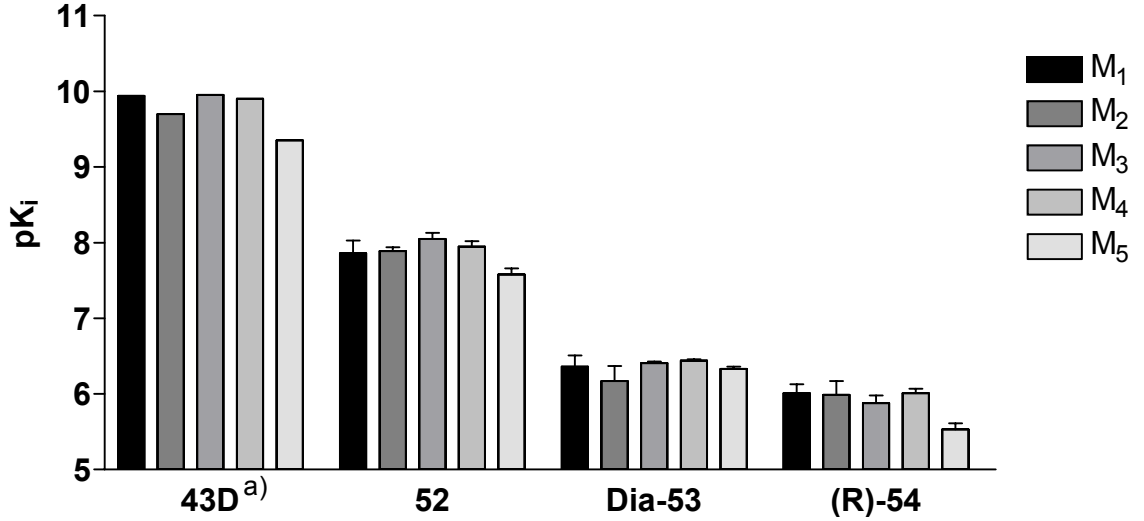


Fig. 4.23 *Affinity values (pK_i) at M_{1-5} receptors of the parent compound **43D**, the dimerised compound **52** and precursors **Dia-53** and **(R)-54**. [a)] Values were measured by K. Kreutzmann (personal communication).*

4.5.4 Tiotropium / glycopyrronium hybrids

The two hybrid molecules of tiotropium and glycopyrronium, **(R)-55** and **(R)-56**, both displayed high affinities at muscarinic subtypes in the range of **43D**. Data are given in Table 4.9 and illustrated in Fig. 4.24.

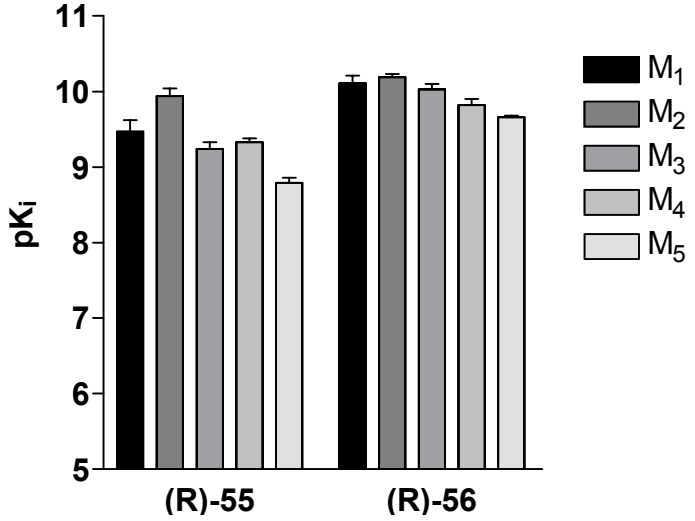


Fig. 4.24 *Affinity values (pK_i) of tiotropium / glycopyrronium hybrids **(R)-55** and **(R)-56** at M_{1-5} receptors.*

4.6 Characterisation of [^{3}H](3R, 2'R)-glycopyrronium

A complete characterisation of [^{3}H](3R, 2'R)-glycopyrronium ([^{3}H]43D) was performed at muscarinic M_{1-5} receptors. This included saturation, competition and kinetic binding experiments.

4.6.1 Saturation binding experiments

To determine the affinity (K_D value) of the labelled compound at M_{1-5} receptors saturation binding experiments were carried out. The calculated K_D values obtained are given in Table 4.10. Saturation binding isotherms and the corresponding Scatchard-plots are given in Fig. 4.25 for M_{1-3} and in Fig. 4.26 for M_{4+5}. Very high affinities (K_D) were measured ranging from 216 to 28 pM matching 9.67 - 10.55 as logarithmic values. No high selectivity for a special subtype was seen on the basis of affinity differences. For all tested subtypes non-specific binding increased linearly within the range of the used concentrations of radioligand and specific binding was saturable and consistent with a one-site binding model. In addition, Scatchard plots were linear indicating a one-site binding model without co-operativity.

Table 4.10 *Equilibrium dissociation constants (K_D) of [^{3}H]43D at hM_{1-5} receptors and the total number of specific binding sites (B_{max}) in membranes from stably transfected CHO-K1 cells derived from saturation experiments.*

Receptor	K_D [nM]	pK_D	B_{max} [fmol/µg protein]
M_1	0.028 ± 0.001	10.55 ± 0.02	2.79 ± 0.22
M_2	0.067 ± 0.007	10.17 ± 0.04	0.18 ± 0.01
M_3	0.028 ± 0.003	10.55 ± 0.05	3.26 ± 0.09
M_4	0.033 ± 0.001	10.46 ± 0.01	0.69 ± 0.05
M_5	0.216 ± 0.041	9.67 ± 0.08	0.30 ± 0.02

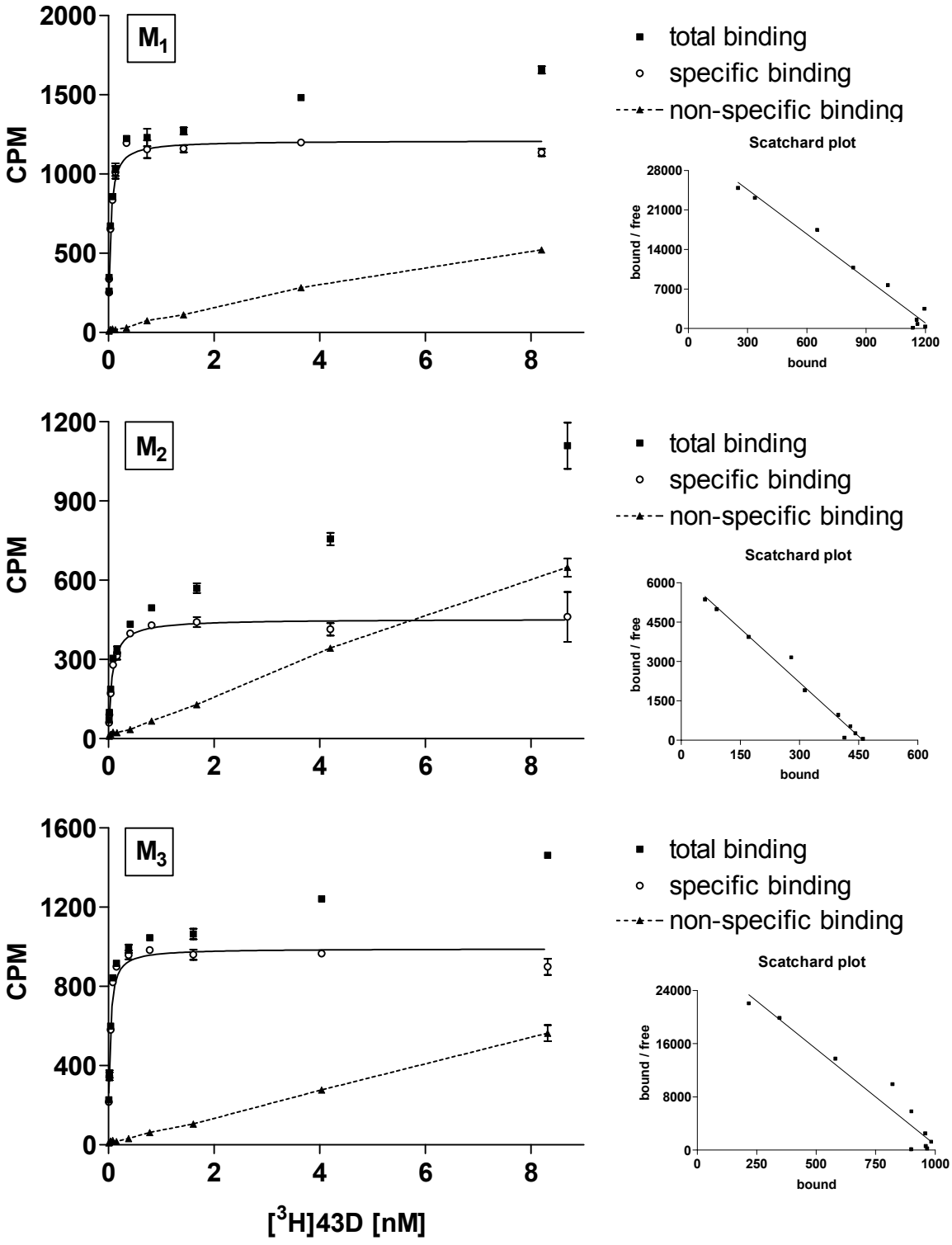


Fig. 4.25 *Saturation binding isotherms displaying total (boxes), specific (circles) and non-specific binding (triangles) of [^{3}H]43D to cloned hM$_{1-3}$ receptors stably expressed in CHO-K1 cells and the corresponding Scatchard plots. Data represent one typical experiment each.*

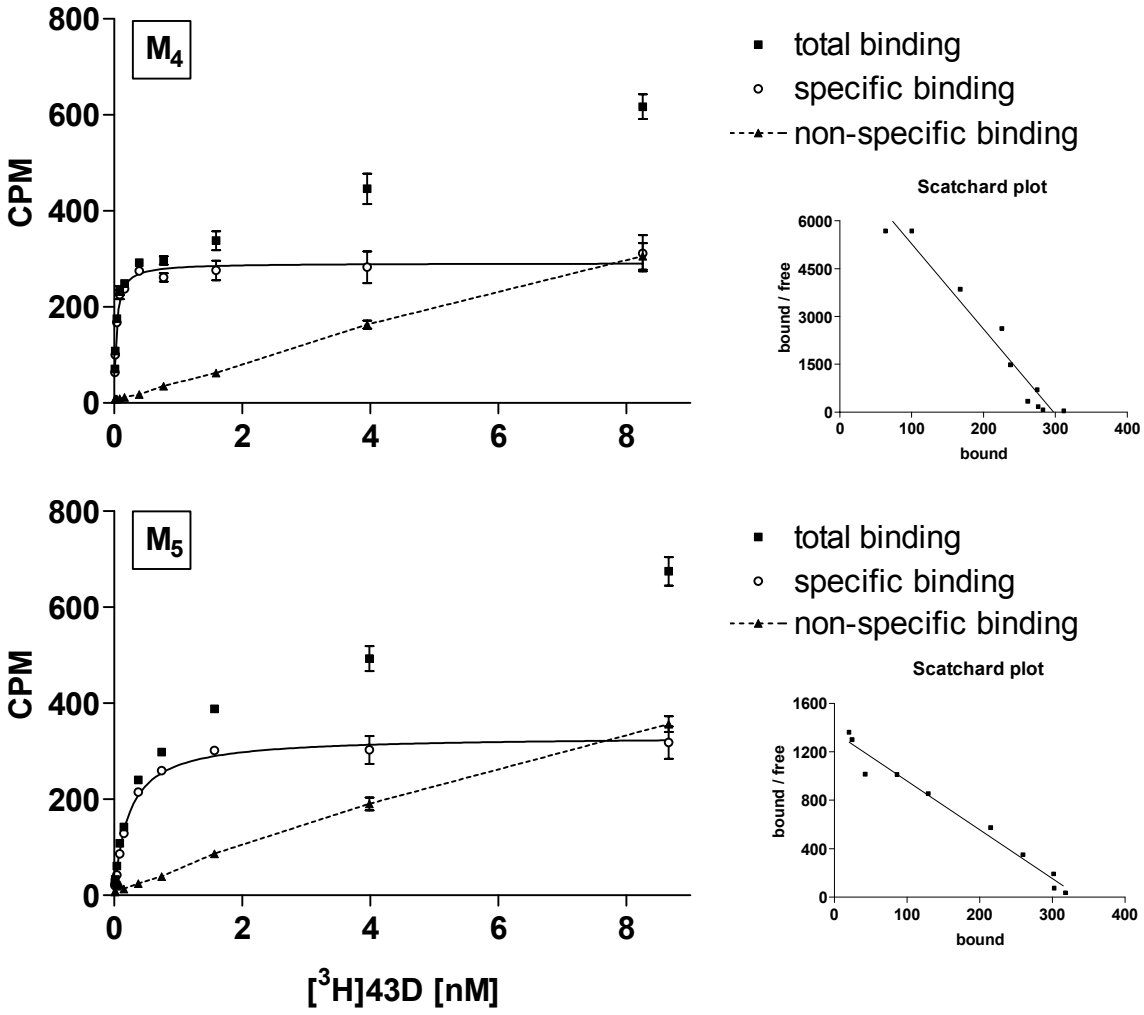


Fig. 4.26 *Saturation binding isotherms displaying total (boxes), specific (circles) and non-specific binding (triangles) of [^{3}H]43D to cloned hM$_{4+5}$ receptors stably expressed in CHO-K1 cells and the corresponding Scatchard plots. Data represent one typical experiment each.*

4.6.2 Competition binding experiments

For further characterisation of [^{3}H]43D competition binding experiments were carried out. The affinity values (pK_i) of examined drugs are given in Table 4.11. The tested drugs were spanning an affinity range of approximately four orders of magnitude and displayed different selectivity profiles. The obtained values are in good agreement with literature data obtained in competition experiments with [^{3}H]NMS (Dörje et al., 1991; Böhme et al., 2003; and unpublished data from our laboratory). Examples of competition curves recorded with [^{3}H]43D are shown in Fig. 4.27.

Table 4.11 *pK_i values and Hill coefficients (in parentheses) of reference drugs at muscarinic receptor subtypes determined in competition binding experiments with [^{3}H]43D. Hill slopes significantly different from unity are given in grey italics.*

	M_1	M_2	M_3	M_4	M_5
Atropine	**8.95** ± 0.08	**8.75** ± 0.07	**9.03** ± 0.05	**9.14** ± 0.07	**8.56** ± 0.02
	(0.92 ± 0.02)	(0.93 ± 0.05)	(0.97 ± 0.09)	(0.96 ± 0.05)	*(0.83 ± 0.02)*
Himbacine	**6.81** ± 0.05	**8.20** ± 0.05	**6.95** ± 0.04	**7.76** ± 0.04	**6.08** ± 0.04
	(1.17 ± 0.14)	(0.94 ± 0.04)	(1.07 ± 0.02)	(1.06 ± 0.12)	(1.02 ± 0.03)
R-Dimethindene	**5.94** ± 0.04	**6.05** ± 0.04	**5.70** ± 0.03	**5.72** ± 0.01	**5.51** ± 0.04
	(1.17 ± 0.10)	(1.17 ± 0.08)	(*1.24 ± 0.01*)	(1.23 ± 0.12)	(*1.14 ± 0.01*)
S-Dimethindene	**6.94** ± 0.04	**7.74** ± 0.04	**7.10** ± 0.01	**6.75** ± 0.06	**6.06** ± 0.05
	(1.02 ± 0.05)	(1.08 ± 0.07)	(1.10 ± 0.05)	(1.10 ± 0.07)	(1.05 ± 0.08)
Pirenzepine	**8.14** ± 0.05	**6.67** ± 0.05	**6.93** ± 0.05	**7.57** ± 0.05	**6.70** ± 0.04
	(0.82 ± 0.05)	(0.95 ± 0.01)	(*0.90 ± 0.04*)	(0.97 ± 0.05)	(*0.86 ± 0.03*)
HHSiD	**7.81** ± 0.03	**7.09** ± 0.04	**7.99** ± 0.05	**7.70** ± 0.03	**6.86** ± 0.04
	(1.05 ± 0.01)	*(0.90 ± 0.01)*	(0.90 ± 0.06)	(0.96 ± 0.06)	(0.94 ± 0.03)

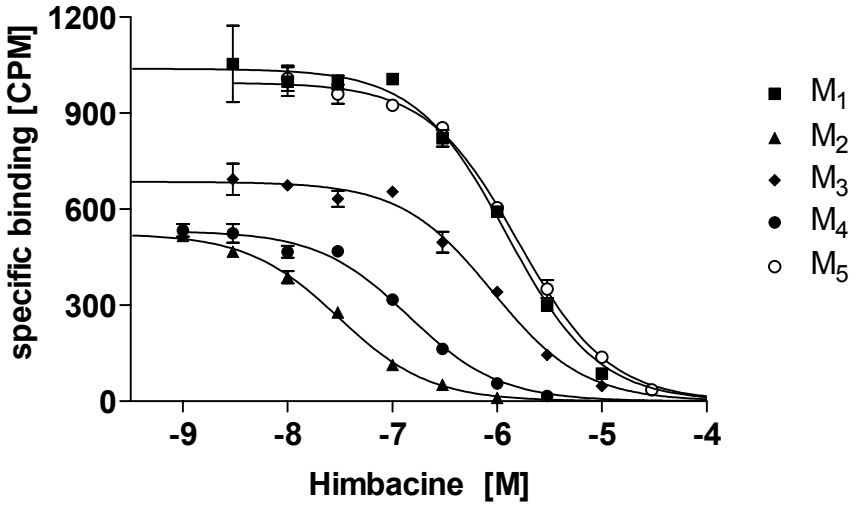


Fig. 4.27 *Competition curves of the M_2 preferring antagonist himbacine at [^{3}H]43D binding sites in membrane preparations from CHO-K1 cells stably expressing hM_{1-5} receptors.*

As an important experiment competition binding studies were carried out using "cold" **43D** as a competitor for the radiolabelled, "hot" compound [^{3}H]43D. Data are given in

Table 4.12 and competition curves are shown in Fig. 4.28. The obtained pK_i values were 2-fold lower at all subtypes compared to the radioligands K_D.

Table 4.12 *pK_i values and Hill coefficients (in parentheses) of **43D** at muscarinic receptor subtypes determined in competition binding experiments with [^{3}H]43D.*

	M_1	M_2	M_3	M_4	M_5
43D	**10.24** ± 0.09	**9.92** ± 0.10	**10.25** ± 0.09	**10.19** ± 0.08	**9.46** ± 0.04
	(1.03 ± 0.10)	(1.03 ± 0.08)	(0.99 ± 0.07)	(0.97 ± 0.01)	(1.01 ± 0.08)

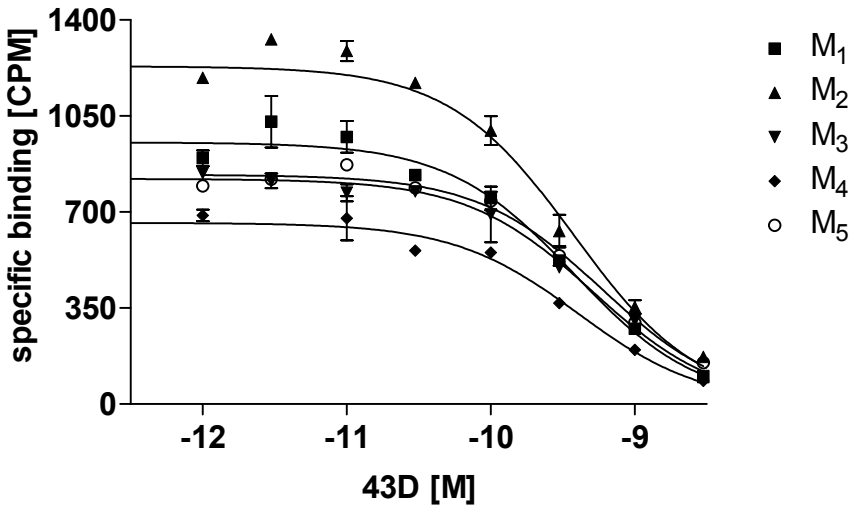


Fig. 4.28 *Competition curves of **43D** versus [^{3}H]43D in membrane preparations from CHO-K1 cells stably expressing $hM_{1\text{-}5}$ receptors.*

4.6.3 Kinetic binding experiments

Association binding experiments were carried out to measure the observed rate constant (k_{obs}) and to calculate the association and dissociation rate constant (k_{on} and k_{off}). A representative example of a set of association curves for the M_5 subtype is given in Fig. 4.29. An increase in B_{max} values (fitted as plateau of curves at equilibrium) can be seen with increasing concentration of radioligand. One can notice that with an increase in radioligand concentration the time needed to reach an equilibrium state decreased.

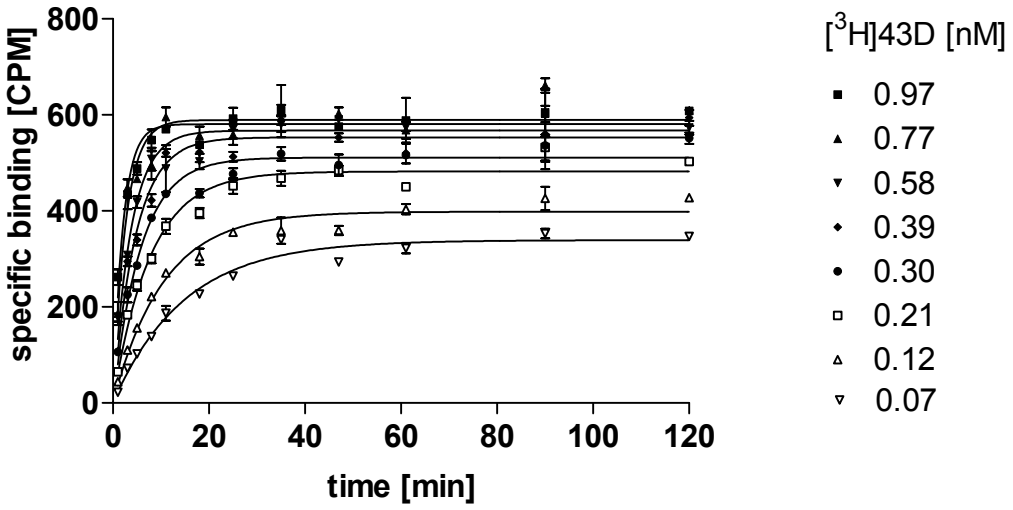


Fig. 4.29 *Association curves recorded with [^{3}H]43D at muscarinic M_5 receptor stably expressed in CHO-K1 cells.*

The fitted values for k_{obs} were plotted versus radioligand concentration and a linear regression procedure was used to fit a straight line as described in material and methods. The slope of the fitted straight line equals the association rate constant (k_{on}). The dissociation rate constant (k_{off}) can be taken from the intersection with the y-axis. The values derived from Fig. 4.29 are given in Fig. 4.30 as an example.

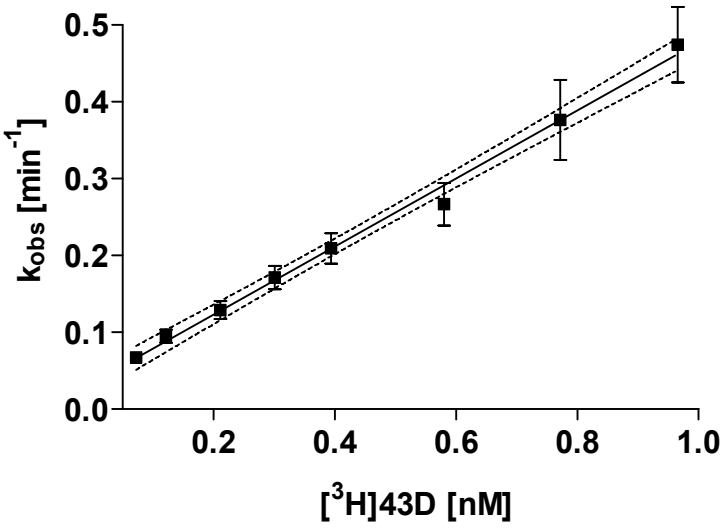


Fig. 4.30 *Linear regression of observed rate constant values (k_{obs}) versus radioligand concentration recorded with [^{3}H]43D at muscarinic M_5 receptor stably expressed in CHO-K1 cells.*

To ensure validity of kinetic data, dissociation binding experiments were additionally conducted at the M_1 and M_2 subtype. Fig. 4.31 gives an example of a dissociation experiment carried out at the M_2 subtype.

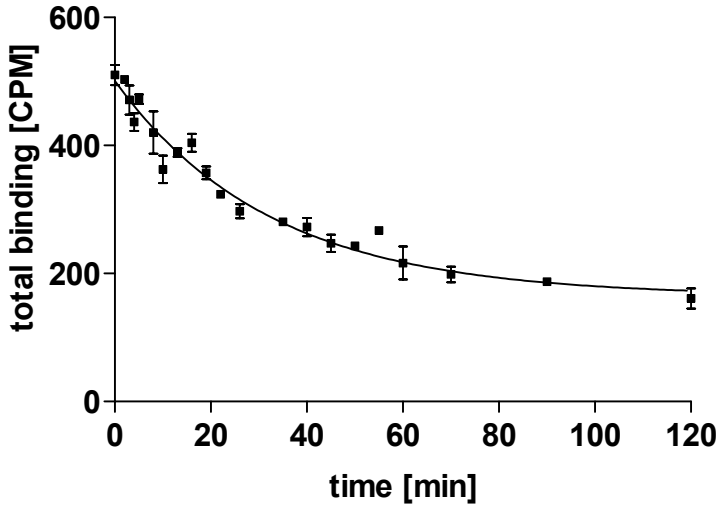


Fig. 4.31 *Dissociation curve of [^{3}H]43D recorded at muscarinic M_2 receptors stably expressed in CHO-K1 cells. Dissociation was initiated by addition of 1 µm atropine.*

A summary of all kinetic data is presented in Table 4.13. Association rate constant (k_{on}), dissociation rate constant (k_{off}) and the corresponding dissociation half lives ($t_{1/2}$) are given. In addition the kinetic K_D ($kinK_D$) was calculated as described in materials and methods.

Table 4.13 *Kinetic constants (k_{on}, k_{off}), the derived dissociation half lives ($t_{1/2}$) and kinetic K_D values of [^{3}H]43D at muscarinic receptor subtypes determined in kinetic binding experiments in membrane preparations of CHO-K1 cells stably expressing hM_{1-5} receptors.*

	M_1	M_2	M_3	M_4	M_5
k_{on} [$nM^{-1}min^{-1}$]	0.337 ± 0.099	0.464 ± 0.093	0.160 ± 0.012	0.229 ± 0.022	0.032 ± 0.009
k_{off} [min^{-1}]	0.010 ± 0.001	0.030 ± 0.004	0.005 ± 0.002	0.009 ± 0.002	0.007 ± 0.002
$t_{1/2}$ [min]	71	23	145	77	100
$kinK_D$ [nM]	0.029 ± 0.003	0.065 ± 0.009	0.031 ± 0.013	0.040 ± 0.011	0.240 ± 0.137
- log($kinK_D$)	10.54 ± 0.05	10.19 ± 0.06	10.55 ± 0.22	10.41 ± 0.13	9.66 ± 0.24

Bar graphs showing the determined association rate constant (k_{on}) at M_{1-5} are given in Fig. 4.32. Calculated dissociation rate constants (k_{off}) are displayed in Fig. 4.33 (**A**), the corresponding dissociation half lives in Fig. 4.33 (**B**). The shortest dissociation half live was obtained at the M_2 subtype with 23 min and the longest $t_{1/2}$ at M_3 with 145 min. This equals a more than 6-fold slower dissociation at M_3 in comparison to M_2.

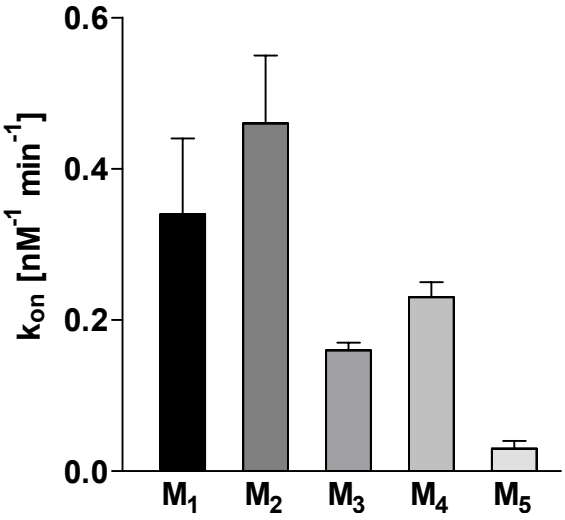


Fig. 4.32 *Association rate constants (k_{on}) of [^{3}H]43D determined in kinetic binding experiments at hM_{1-5} receptors stably expressed in CHO-K1 cells.*

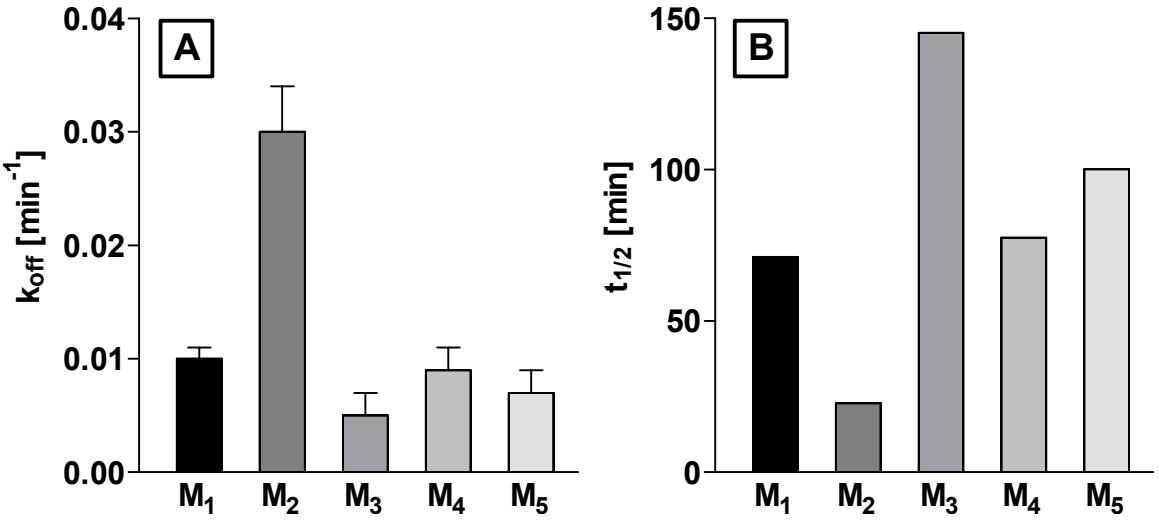


Fig. 4.33 *Dissociation rate constants (k_{off}) of [^{3}H]43D (**A**) and corresponding dissociation half lives ($t_{1/2}$) (**B**) determined in kinetic binding experiments at hM_{1-5} receptors stably expressed in CHO-K1 cells.*

4.7 M_2-selective antagonists related to dimethinende

Table 4.14 gives the affinity values (pK_i) for compounds **57** - **83** at $hM_{1\text{-}5}$ and hH_1 receptors.

Table 4.14 *pK_i values and Hill coefficients (in parentheses) of dimethindene (**57A**) and its analogues **57B** - **83** determined in radioligand binding studies at $hM_{1\text{-}5}$ and hH_1 receptors. Hill slopes significantly different from unity are given in grey italics.*

No.	M_1	M_2	M_3	M_4	M_5	H_1
(±)-57A	**6.43** ± 0.03	**7.25** ± 0.03	**6.58** ± 0.04	**6.26** ± 0.05	**5.98** ± 0.02	**9.16** ± 0.12
	(1.04 ± 0.02)	(0.98 ± 0.04)	(0.98 ± 0.07)	(1.02 ± 0.05)	(0.97 ± 0.06)	(1.07 ± 0.05)
(+)-57A	**6.72** ± 0.05	**7.52** ± 0.05	**6.86** ± 0.01	**6.53** ± 0.05	**6.12** ± 0.03	**7.16** ± 0.06
	(0.93 ± 0.02)	(1.00 ± 0.04)	(1.02 ± 0.01)	(0.99 ± 0.03)	(1.02 ± 0.05)	(1.04 ± 0.15)
(-)-57A	**5.73** ± 0.03	**5.91** ± 0.05	**5.47** ± 0.04	**5.41** ± 0.01	**5.57** ± 0.03	**9.36** ± 0.14
	(1.01 ± 0.03)	(1.01 ± 0.04)	(1.05 ± 0.07)	(0.99 ± 0.09)	(0.99 ± 0.03)	(1.14 ± 0.24)
57B[a)]	**7.46**	**7.91**	**7.50**	**7.01**	**7.06**	**7.54** ± 0.03
						(0.84 ± 0.23)
58A[a)]	**6.06**	**6.84**	**5.74**	**6.00**	**5.66**	**8.35** ± 0.08
						(0.96 ± 0.10)
58B[a)]	**6.64**	**7.29**	**6.13**	**6.22**	**5.95**	**7.90** ± 0.05
						(1.00 ± 0.08)
59A[a)]	**6.35**	**7.35**	**6.02**	**6.34**	**5.68**	**7.67** ± 0.07
						(1.04 ± 0.04)
59B	**6.66** ± 0.03	**8.00** ± 0.02	**6.54** ± 0.01	**6.95** ± 0.08	**6.38** ± 0.01	**8.20** ± 0.09
	(1.00 ± 0.07)	(1.01 ± 0.08)	(1.01 ± 0.09)	(1.00 ± 0.05)	(0.96 ± 0.10)	(0.99 ± 0.12)
60A[a)]	**5.72**	**6.07**	**5.75**	**5.72**	**5.63**	**8.08** ± 0.06
						(0.95 ± 0.03)
60B	**5.88** ± 0.05	**6.96** ± 0.06	**5.50** ± 0.02	**5.89** ± 0.05	**5.44** ± 0.08	**7.81** ± 0.06
	(0.98 ± 0.04)	(1.06 ± 0.18)	*(0.94 ± 0.02)*	(0.98 ± 0.01)	(1.08 ± 0.10)	(0.91 ± 0.11)
61A[a)]	**5.36**	**5.84**	**5.56**	**5.38**	**5.57**	**7.91** ± 0.05
						(1.09 ± 0.04)
61B	**5.08** ± 0.01	**6.71** ± 0.09	**5.24** ± 0.09	**5.50** ± 0.02	**< 4.5**	**6.31** ± 0.03
	(0.98 ± 0.03)	(1.04 ± 0.03)	(1.14 ± 0.22)	(1.04 ± 0.07)		(1.15 ± 0.12)

Table 4.14 (continued)

No.	M_1	M_2	M_3	M_4	M_5	H_1
62B	**5.01** ± 0.09	**5.99** ± 0.04	**4.88** ± 0.06	**5.12** ± 0.05	**5.37** ± 0.10	**6.17** ± 0.03
	(0.80 ± 0.27)	(0.98 ± 0.11)	(1.01 ± 0.05)	(0.99 ± 0.05)	(1.00 ± 0.06)	(1.21 ± 0.36)
63B	**6.00** ± 0.52	**5.97** ± 0.70	**5.68** ± 0.61	**5.71** ± 0.79	**5.40** ± 0.49	**6.49** ± 0.08
	(1.09 ± 0.28)	(1.28 ± 0.25)	(1.18 ± 0.15)	*(1.16 ± 0.05)*	(1.04 ± 0.05)	(0.98 ± 0.04)
64A	**5.61** ± 0.07	**5.79** ± 0.08	**5.26** ± 0.06	**5.51** ± 0.09	**5.25** ± 0.06	**8.48** ± 0.10
	(0.92 ± 0.08)	*(0.68 ± 0.10)*	(1.07 ± 0.13)	(0.96 ± 0.03)	(1.16 ± 0.09)	(0.98 ± 0.17)
64B	**5.68** ± 0.09	**6.80** ± 0.04	**5.37** ± 0.04	**5.74** ± 0.10	**5.17** ± 0.05	**6.36** ± 0.03
	(0.93 ± 0.04)	(0.98 ± 0.02)	(1.00 ± 0.08)	(1.02 ± 0.02)	(1.12 ± 0.13)	(1.12 ± 0.17)
65B	**5.20** ± 0.09	**6.13** ± 0.06	**5.23** ± 0.04	**5.86** ± 0.06	**5.10** ± 0.05	**5.56** ± 0.10
	(0.83 ± 0.07)	*(0.68 ± 0.09)*	(0.93 ± 0.31)	*(0.66 ± 0.05)*	(0.85 ± 0.07)	(0.93 ± 0.14)
66B	**6.19** ± 0.06	**7.11** ± 0.09	**5.94** ± 0.01	**6.39** ± 0.05	**5.44** ± 0.06	**5.63** ± 0.07
	(1.04 ± 0.02)	(0.95 ± 0.04)	(1.11 ± 0.10)	(1.15 ± 0.08)	(1.05 ± 0.04)	(0.99 ± 0.09)
67B	**5.89** ± 0.22	**5.66** ± 0.11	**5.50** ± 0.23	**5.97** ± 0.14	**5.22** ± 0.20	**< 4.5**
	(0.83 ± 0.17)	(0.67 ± 0.30)	(0.92 ± 0.03)	(0.63 ± 0.26)	(0.96 ± 0.01)	
68B	**5.54** ± 0.21	**5.77** ± 0.21	**5.34** ± 0.27	**5.44** ± 0.28	**5.34** ± 0.29	**< 4.5**
	(0.79 ± 0.23)	(0.88 ± 0.23)	(1.10 ± 0.12)	(1.02 ± 0.10)	(1.22 ± 0.17)	
69B	**< 4.5**	**4.80** ± 0.37	**< 4.5**	**< 4.5**	**< 4.5**	**< 4.5**
		(0.93 ± 0.12)				
70B	**5.68** ± 0.13	**6.14** ± 0.08	**6.00** ± 0.05	**5.80** ± 0.14	**5.37** ± 0.08	**6.46** ± 0.10
	(0.97 ± 0.06)	(1.04 ± 0.07)	*(1.10 ± 0.04)*	*(0.86 ± 0.04)*	(1.05 ± 0.02)	*(1.18 ± 0.06)*
71B	**6.27** ± 0.13	**7.57** ± 0.02	**6.36** ± 0.05	**6.71** ± 0.05	**6.05** ± 0.05	**6.30** ± 0.04
	(1.01 ± 0.09)	(0.95 ± 0.12)	(1.01 ± 0.07)	(1.01 ± 0.02)	(1.07 ± 0.05)	(1.05 ± 0.05)
(±)-72B	**6.51** ± 0.08	**8.47** ± 0.13	**6.29** ± 0.03	**6.77** ± 0.03	**6.40** ± 0.05	**5.30** ± 0.04
	(0.95 ± 0.03)	(0.88 ± 0.08)	(1.05 ± 0.01)	(1.19 ± 0.10)	(1.03 ± 0.06)	*(1.46 ± 0.08)*
(+)-72B	**6.69** ± 0.11	**8.59** ± 0.10	**6.58** ± 0.00	**7.10** ± 0.10	**6.51** ± 0.07	**5.46** ± 0.02
	(1.08 ± 0.06)	(0.78 ± 0.24)	*(1.06 ± 0.02)*	(0.92 ± 0.04)	(0.97 ± 0.04)	(1.03 ± 0.06)
(-)-72B	**6.21** ± 0.10	**7.33** ± 0.08	**6.29** ± 0.09	**6.34** ± 0.04	**5.73** ± 0.07	**6.13** ± 0.04
	(0.95 ± 0.06)	(0.73 ± 0.29)	(1.03 ± 0.10)	(1.03 ± 0.12)	(1.15 ± 0.14)	(1.04 ± 0.04)
73B	**6.34** ± 0.08	**7.57** ± 0.16	**6.34** ± 0.04	**6.86** ± 0.08	**5.92** ± 0.13	**5.09** ± 0.09
	(1.03 ± 0.06)	(1.00 ± 0.39)	(1.02 ± 0.05)	(1.04 ± 0.06)	(1.00 ± 0.03)	(0.89 ± 0.12)
74B	**< 4.5**	**5.22** ± 0.10	**< 4.5**	**4.55** ± 0.04	**< 4.5**	**< 4.5**
		(1.03 ± 0.22)		(1.09 ± 0.44)		

Table 4.14 (continued)

No.	M_1	M_2	M_3	M_4	M_5	H_1
(±)-75A[a)]	**6.13**	**7.60**	**5.65**	**6.13**	**5.66**	**6.96** ± 0.04 (0.99 ± 0.02)
(+)-75A[a)]	**5.23**	**6.24**	**4.68**	**5.26**	**5.29**	**6.93** ± 0.07 (0.98 ± 0.05)
(-)-75A[a)]	**5.81**	**7.37**	**5.39**	**5.75**	**4.93**	**5.79** ± 0.03 (0.93 ± 0.09)
76B	**5.84** ± 0.06 (1.05 ± 0.03)	**6.76** ± 0.05 (1.04 ± 0.06)	**5.65** ± 0.08 (0.96 ± 0.20)	**6.03** ± 0.08 (0.95 ± 0.14)	**5.20** ± 0.04 (1.01 ± 0.05)	**5.69** ± 0.04 (0.93 ± 0.12)
77B	**6.84** ± 0.07 (0.98 ± 0.06)	**7.82** ± 0.20 (1.01 ± 0.09)	**6.53** ± 0.03 (0.99 ± 0.06)	**7.14** ± 0.05 (1.01 ± 0.06)	**6.17** ± 0.10 (1.02 ± 0.01)	**5.85** ± 0.05 (1.11 ± 0.07)
78B	**6.42** ± 0.04 (1.02 ± 0.04)	**7.99** ± 0.13 (0.87 ± 0.15)	**6.10** ± 0.02 (1.02 ± 0.07)	**6.71** ± 0.05 (1.05 ± 0.07)	**6.12** ± 0.05 *(1.38 ± 0.12)*	**5.72** ± 0.09 (1.30 ± 0.28)
79B	**6.86** ± 0.10 (1.21 ± 0.16)	**8.22** ± 0.04 (0.76 ± 0.18)	**6.60** ± 0.10 (1.08 ± 0.16)	**7.24** ± 0.05 (1.00 ± 0.11)	**6.14** ± 0.11 (1.00 ± 0.09)	**5.35** ± 0.07 (1.09 ± 0.11)
80B	**6.63** ± 0.07 *(1.33 ± 0.11)*	**7.61**± 0.06 (1.05 ± 0.06)	**6.29** ± 0.03 (1.53 ± 0.22)	**6.85** ± 0.06 *(1.41 ± 0.05)*	**6.07** ± 0.05 *(1.41 ± 0.14)*	**6.14** ± 0.06 (1.18 ± 0.14)
81B	**6.98** ± 0.04 (1.15 ± 0.09)	**7.68** ± 0.04 (0.99 ± 0.06)	**6.70** ± 0.06 (0.99 ± 0.06)	**7.27** ± 0.03 (1.04 ± 0.01)	**6.16** ± 0.09 (1.10 ± 0.11)	**5.66** ± 0.05 (0.97 ± 0.06)
82B	**6.67** ± 0.06 (1.01 ± 0.05)	**7.96** ± 0.14 (0.98 ± 0.15)	**6.49** ± 0.01 (1.04 ± 0.00)	**7.00** ± 0.07 (1.05 ± 0.02)	**6.52** ± 0.03 *(1.24 ± 0.12)*	**5.66** ± 0.01 (1.00 ± 0.05)
83B	**6.78** ± 0.05 (1.08 ± 0.03)	**7.53** ± 0.10 (1.06 ± 0.39)	**6.48** ± 0.03 (1.03 ± 0.01)	**7.08** ± 0.05 (1.03 ± 0.01)	**6.17** ± 0.03 (1.03 ± 0.02)	**5.70** ± 0.03 (1.02 ± 0.02)

[a)] *Data at muscarinic receptor subtypes measured by Dr. C. Keim and K. Kreutzmann (Böhme et al., 2003).*

4.7.1 Compounds with modifications in side chain length and amino moiety

Several pairs of compounds were tested with reduced side chain length (n=1) in comparison to their congeners with a two carbon side chain (n=2). Compounds **57A/B** and **58A/B** served as starting points. We saw increased affinities at muscarinic receptor subtypes and at the same time reduced affinty at H_1 receptors for compounds with n=1. Data are given in Table 4.14. Bar graphs showing affinities

for compounds **57A/B** - **59A/B** are given in Fig. 4.34 (**A** + **B**). Changes resulting from reduction of chain length are shown in Fig. 4.34 (**C**). The most difficult problem in former studies was to increase absolute affinity at muscarinic M_2 subtypes. To find out whether the findings with at **57A/B** and **58A/B** were a coincidence or a "rule", we synthesised several other derivatives with reduced chain length (n=1). In the case of the parent compound **57A** reduction of chain length to **57B** led to an increase of approximately one order of magnitude at muscarinic receptor subtypes and to a 42-fold reduced affinity at H_1. A 3-fold increase was found for compounds **58A/B** and also a less pronounced reduction at H_1 (3-fold). For analogues **59A/B** an increase in affinity at $M_{1\text{-}5}$ and H_1 was observed. For compounds **57A/B** - **59A/B** an increase in affinity at muscarinic receptors was found at all subtypes. **59B** conserved the subtype selectivity found for **59A** and displayed 11-fold selectivity for M_2 but with higher affinity (pK_i = 8.00 and 7.35, respectively). A problem was the even higher H_1 affinity.

Additional derivatives were examined. Fig. 4.35 gives the affinity data for **60A/B**, **61A/B**, displaying cyclic amino moieties and **64A/B** with an aromatic substituent. Interestingly, for these compounds a pronounced increase in affinity at muscarinic receptor subtypes was only found for M_2 (8-, 7-, 10-fold, respectively). At the same time, H_1 affinity was reduced for **60A/B** (2-fold), **61A/B** (40-fold) and drastically reduced for **64A/B** (132-fold). Reducing side chain length resulted in compounds displaying good subtype selectivity (at least 12-, 16-, 11-fold) in favour for the M_2 subtype. However, absolute affinities were not sufficiently high (pK_i = 6.96, 6.71 and 6.80, respectively). For **61B** and **64B**, M_2 affinity was a little higher than at H_1. Compounds **62B** and **63B** showed low affinities (pK_i < 6.0) at $M_{1\text{-}5}$ and poor (4-fold) or no subtype selectivity. Additionally, H_1 affinities were higher than at M_2 (Fig. 4.36).

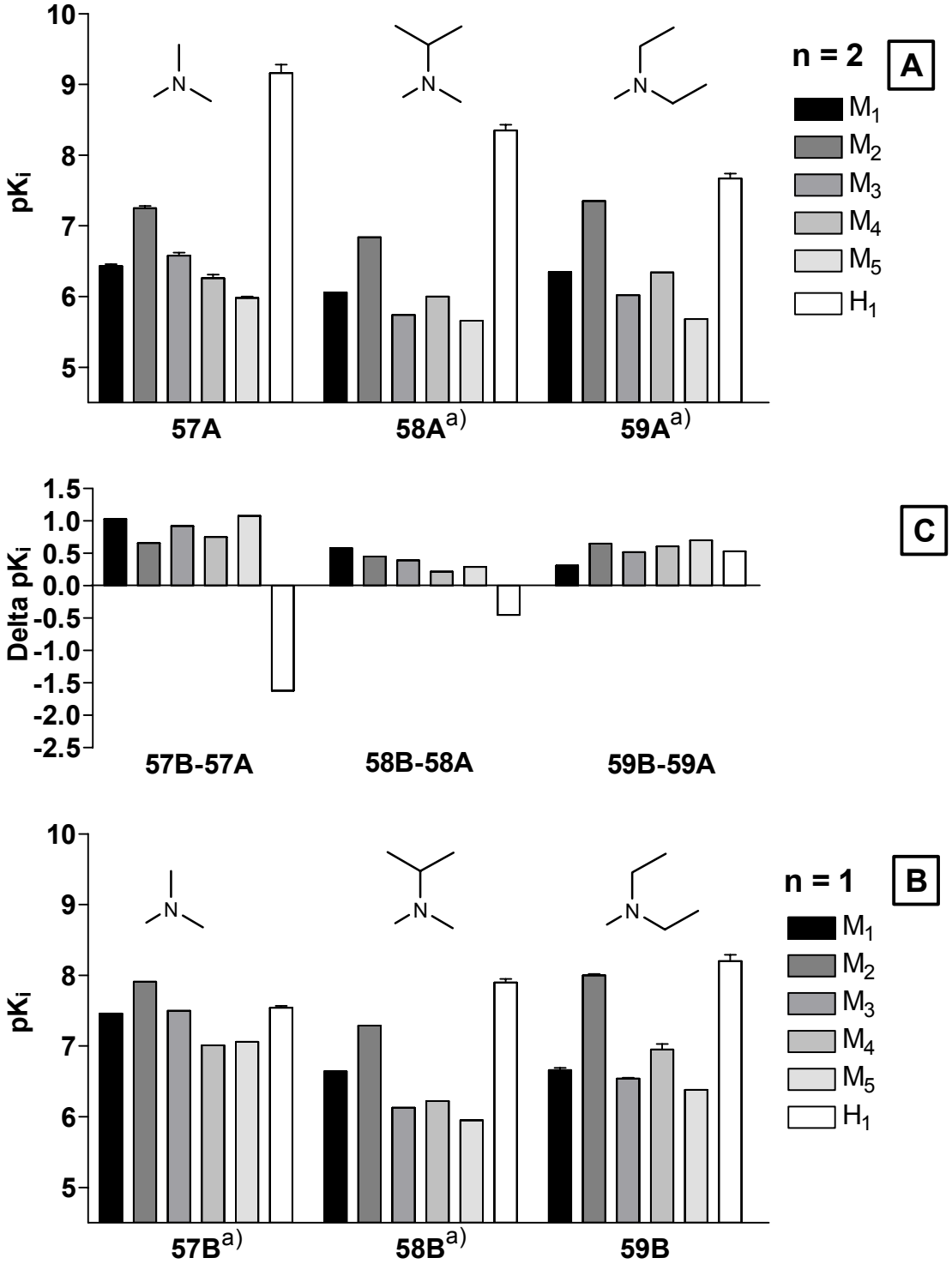


Fig. 4.34 *Affinity values (pK_i) of compounds with modifications in the amino moiety and with differing side chain length; compounds **57A** - **59A** with n=2 (**A**), **57B** - **59B** with n=1 (**B**) and comparison of resulting changes in affinities (**C**) at M_{1-5} and H_1 receptors.* [a)] *Data at muscarinic receptor subtypes taken from Böhme et al. (2003).*

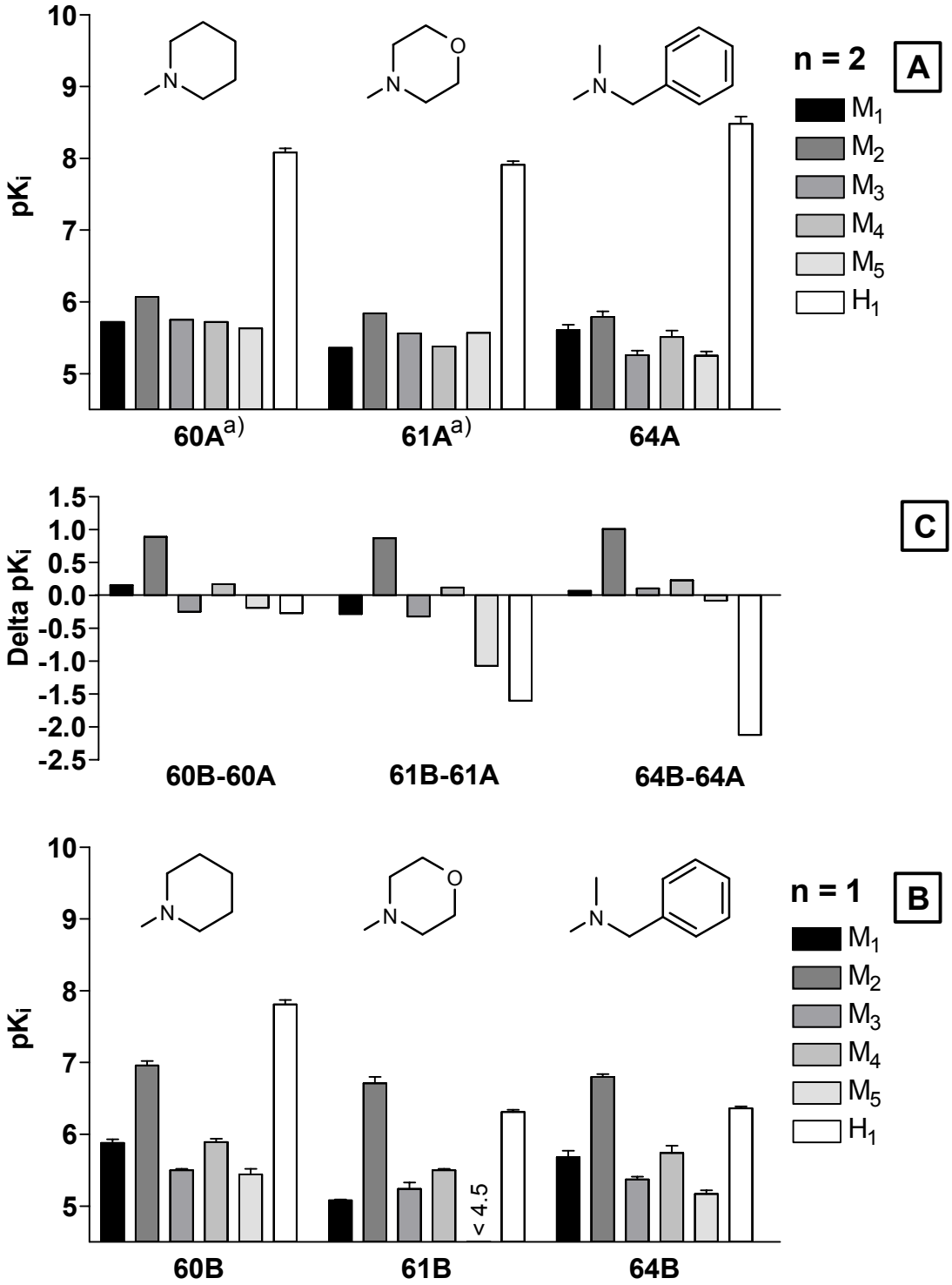


Fig. 4.35 *Affinity values (pK_i) of compounds with modifications in the amino moiety and with differing side chain length; compounds **60A**, **61A** and **64A** with n=2 (**A**), **60B**, **61B** and **64B** with n=1 (**B**) and comparison of resulting changes in affinities (**C**) at $M_{1\text{-}5}$ and H_1 receptors. [a)] Data at muscarinic receptor subtypes taken from Böhme et al. (2003).*

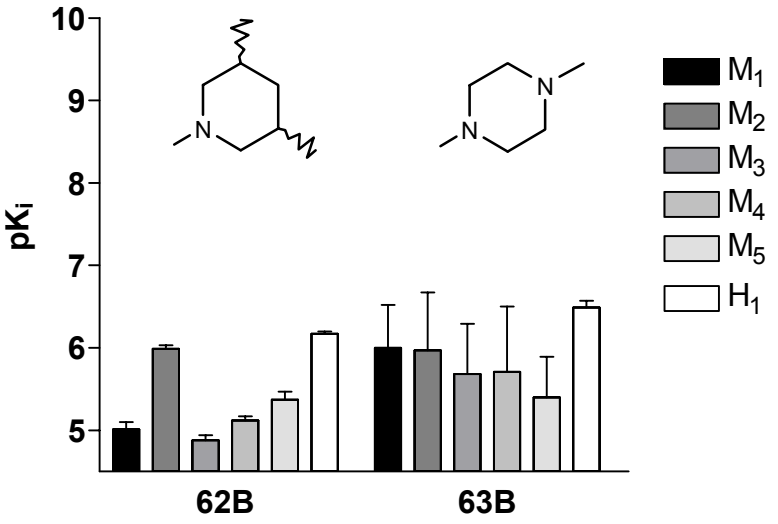


Fig. 4.36 *Affinity values (pK_i) of compounds **62B** and **63B** (n=1) and modifications in the amino moiety at M_{1-5} and H_1 receptors.*

Taken together, it was shown that in all cases affinity to muscarinic receptor subtypes was improved by reducing side chain length (in cases of **60A/B**, **61A/B** and **64A/B**, this gain in affinity was exclusively found at M_2) and simultaneously (with exception of **59A/B**) a decrease in H_1 affinity was achieved. Therefore, synthesis was concentrated on compounds with reduced side chain length.

4.7.2 Compounds with a benzyl or phenylethyl substituent at the basic nitrogen

Several analogues with reduced side chain length were synthesised with a benzyl or phenylethyl group affixed at the basic nitrogen. It was found that the phenylethyl-substituted compounds **70B**, **72B**, **74B** had higher affinities at muscarinic receptor subtypes in comparison to their benzyl congeners **65B**, **66B**, **69B** (see Fig. 4.37). The ispopropyl group in **72B** was found as an optimum, as minor changes in this part of the molecule resulted in decreased affinity and selectivity (compared to **70B**, **71B**, **73B**, **74B**; see Fig. 4.37 (**B**) and Fig. 4.38).

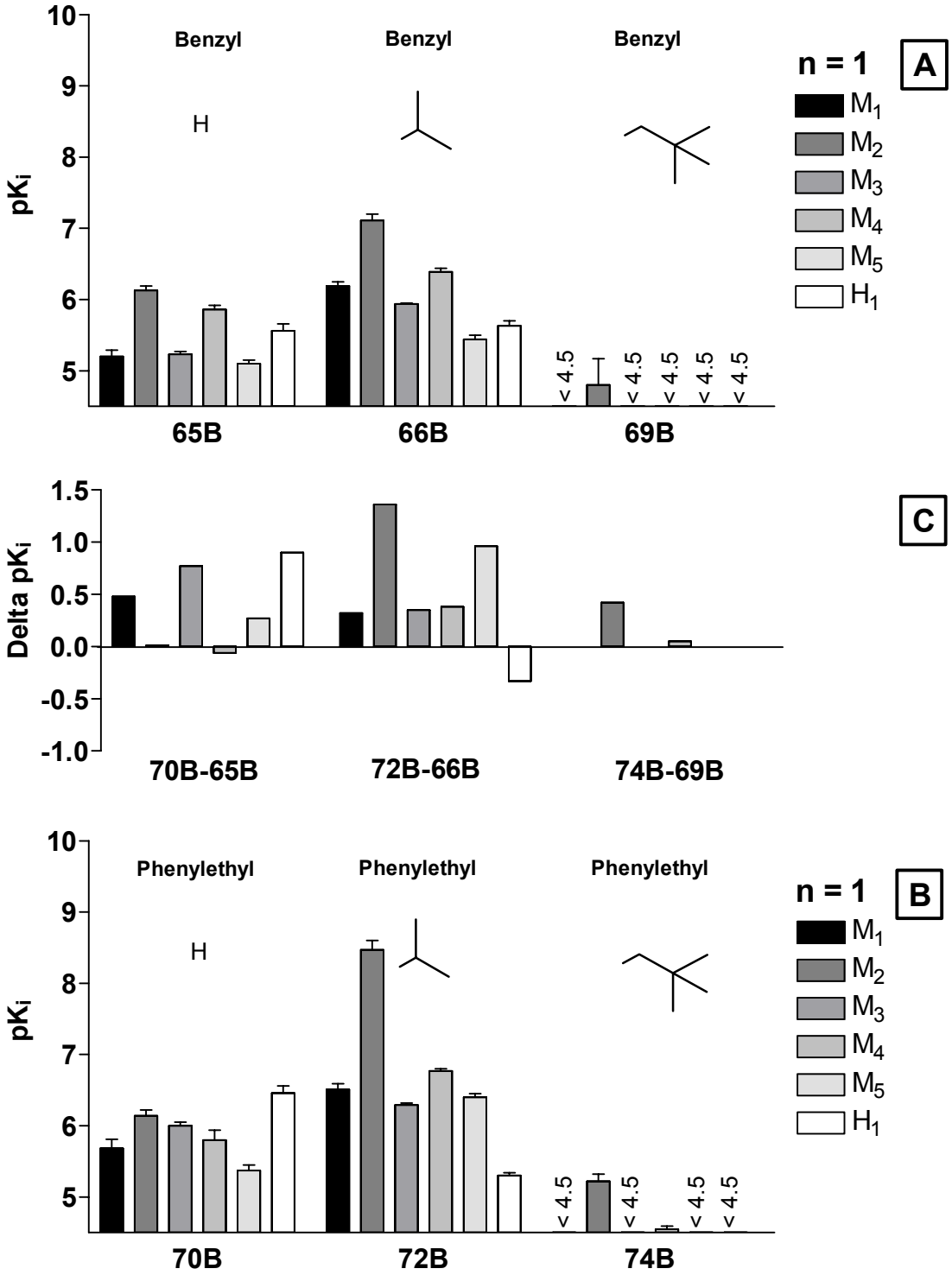


Fig. 4.37 *Affinity values (pK_i) of compounds with a benzyl substituent (**65B**, **66B**, **69B** (**A**)), or a phenylethyl substituent (**70B**, **72B**, **74B** (**B**)) at the amino moiety and comparison of resulting changes in affinities (**C**) at $M_{1\text{-}5}$ and H_1 receptors.*

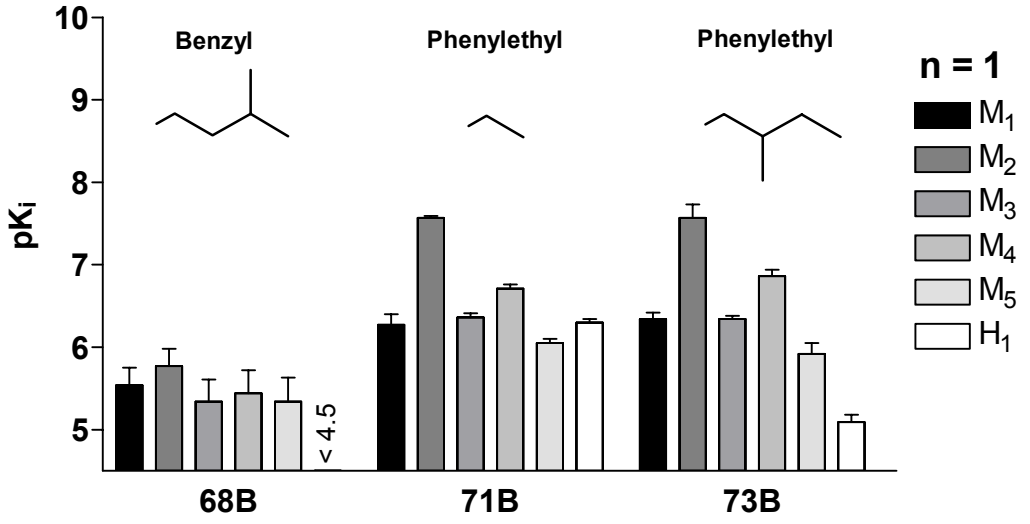


Fig. 4.38 *Affinity values (pK_i) of compounds with a benzyl (**68B**) or a phenylethyl substituent (**71B** and **73B**) in the amino moiety at M_{1-5} and H_1 receptors.*

4.7.3 Pure enantiomers

The parent compound **57A**, the diisopropyl congener **75A** and the most interesting compound of our new series, **72B**, were isolated as pure enantiomers. Binding data of enantiomers are given in Table 4.14. Affinity data for **(±)-57A** and its enantiomers (Fig. 4.39) were in good agreement with those published (Böhme et al., 2003). For this and the following two figures the eutomer at muscarinic receptor subtypes is shown to the left of the racemic mixture (middle), the distomer on the right hand side. **(±)-57A** showed only little subtype selectivity for M_2 (5-fold to 19-fold) and a 81-fold higher affinity to H_1 (pK_i = 9.16). As previously shown in literature (see also 5.4.3.5), inverse stereoselectivity was found concerning M_{1-5} and H_1 subtypes, respectively. **(+)-57A** was the eutomer at muscarinic receptor subtypes with an approximately 2-fold higher affinity at muscarinic receptor subtypes and a 100-fold lower affinity at H_1 receptors than the racemate. **(-)-57A** is the distomer at M_{1-5}, but the eutomer at H_1. Taken together, **(+)-57A** has a 5 - 25-fold selectivity for M_2 receptors, but only a 2-fold specificity, as far as H_1 receptors are concerned.

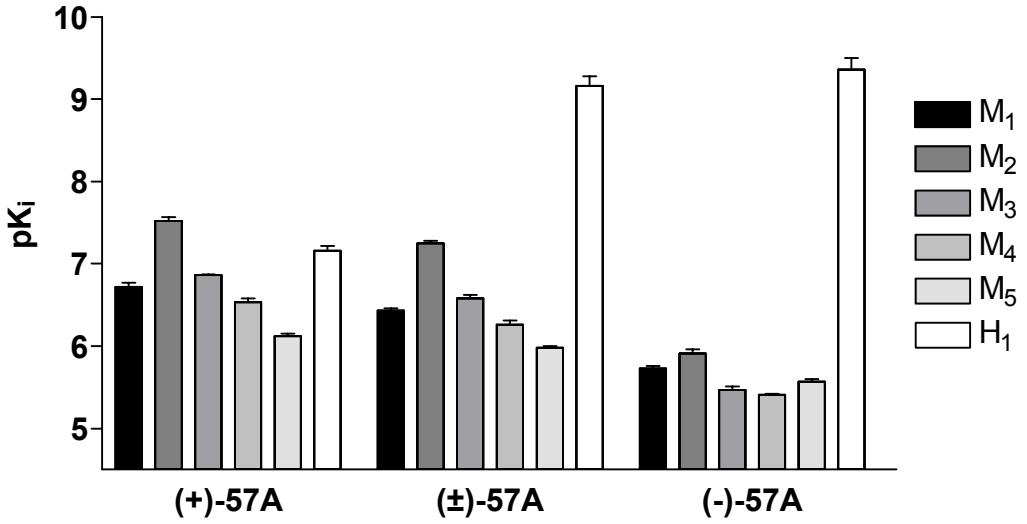


Fig. 4.39 *Affinity values (pK_i) of* ***(±)-57A*** *and its enantiomers at M_{1-5} and H_1 receptors.*

Previous SAR studies in our laboratory were done in order to increase selectivity, specifity and absolute affinity at M_2 receptors. We found compound **(±)-75A** to possess better selectivity and specificity than the parent compound **(±)-57A**. The eutomer at M_{1-5}, **(-)-75A**, had good M_2 selectivity (36 - 275-fold) and a 38-fold specificity versus H_1 (Fig. 4.40). Racemic **(±)-75A** and both enantiomers were M_2-selective compounds. However, the absolute affinity at M_2 was not increased in comparison to **(+)-57A** (pK_i = 7.37 and 7.52, respectively).

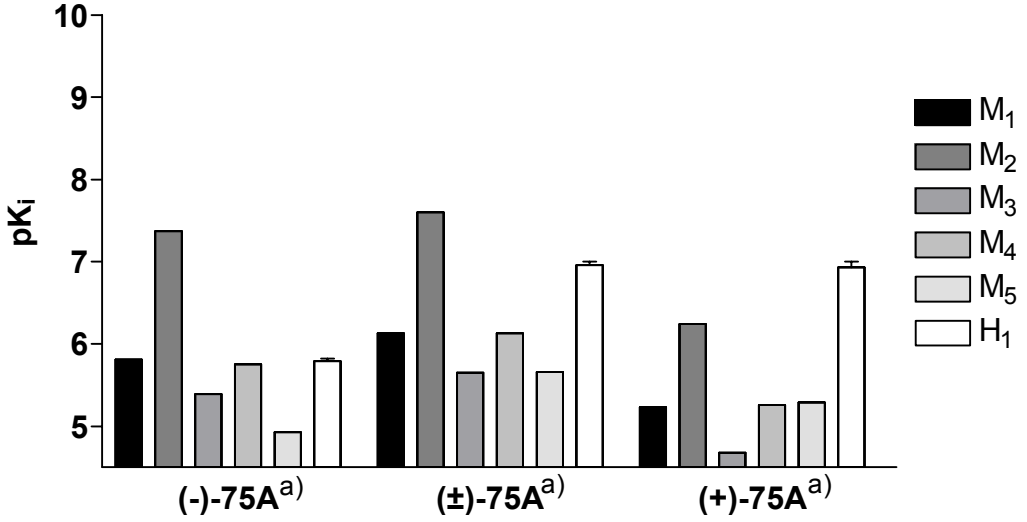


Fig. 4.40 *Affinity values (pK_i) of* ***(±)-75A*** *and its enantiomers at hM_{1-5} and hH_1 receptors.* [a)] *Data taken from Böhme et al. (2003).*

As shown in Fig. 4.37, we found **(±)-72B** to possess high affinity at M_2 (pK_i = 8.47), comparable selectivity to **(-)-75A** and improved specificity. Fig. 4.41 summarizes the binding data for the tested enantiomers. **(+)-72B** was the eutomer at M_{1-5}. It is a highly potent M_2 antagonist displaying good selectivity M_2/M_1: 79-fold; M_2/M_3: 102-fold; M_2/M_4: 31-fold; M_2/M_5: 120-fold and very high specificity M_2/H_1 1349-fold.

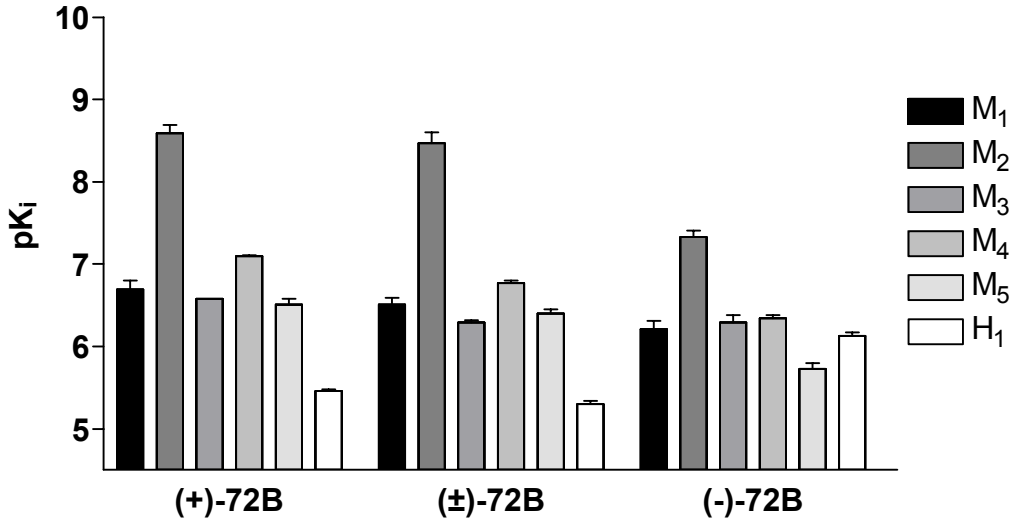


Fig. 4.41 *Affinity values (pK_i) of **(±)-72B** and its enantiomers at M_{1-5} and H_1 receptors.*

4.7.4 Compounds related to 72B

(+)-72B seems to have good binding characteristics to quantify M_2 receptors in AD patients with PET. Additional derivatives of **72B** were synthesised to find a congener displaying comparable binding data with an appropriate substituent for radioactive labelling. Usually an ^{18}F or ^{11}C atom is inserted into a molecule for PET-studies. Exchanging the isopropyl group of **72B** by a fluoroethyl group led to **76B**. Introducing a methyl group in para-position of the phenylethyl substituent of **72B** resulted in **77B**. Binding data for compounds **76B** and **77B** are given in Table 4.14, and are illustrated in Fig. 4.42. Even the minor changes from **72B** → **76B** resulted in a dramatic decrease in affinity at muscarinic receptor subtypes (51-fold at M_2). Insertion of a para-methyl group (**72B** → **77B**) led to a 4-fold decrease in affinity at M_2 (pK_i = 7.82). As the other subtypes showed a less pronounced decrease in affinity by methyl-substitution, subtype selectivity was decreased (M_2/M_4: 5-fold).

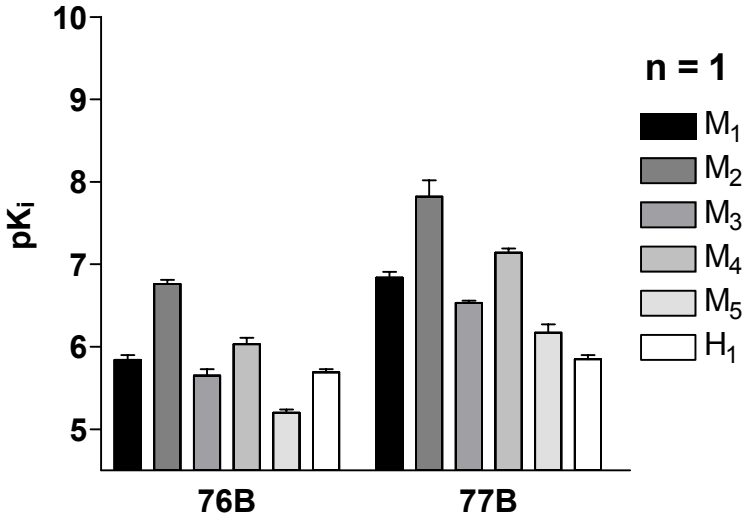


Fig. 4.42 *Affinity values (pK$_i$) of **76B** and **77B** at M$_{1-5}$ and H$_1$ receptors.*

4.7.5 Compounds related to 72B with meta- or para-substituents at the phenylethyl group

Additional derivatives closely related to **72B** with a substituent in meta- or para-position of the aromatic ring at the phenylethyl group were tested. **78B** and **79B** had a methoxy group, **80B** and **81B** a chlorine atom, **82B** and **83B** a fluorine atom. Data are given in Table 4.14. Fig. 4.43 (**A**) shows binding data for compounds with a meta-substituent, Fig. 4.43 (**C**) for the para-congeners, and Fig. 4.43 (**B**) a comparison of these regioisomers. **78B** showed good affinity at M_2 (pK_i = 7.99) and sufficient selectivity (at least 19-fold M_2/M_4) and specificity (186-fold M_2/H_1). The para-substituted congener **79B** had a 2-fold higher affinity at M_2 (pK_i = 8.22), but reduced selectivity (10-fold M_2/M_4), whereas specificity was increased (741-fold M_2/H_1). The chlorine derivatives **80B** and **81B** had lower affinities at M_2 (pK_i = 7.61 and 7.68, respectively), lower selectivity (6-fold and 3-fold M_2/M_4), but good specificity (30-fold and 105-fold M_2/H_1). Concerning **82B**, a good M_2 affinity was seen (pK_i = 7.96), 9-fold selectivity M_2/M_4, and 200-fold specificity (M_2/H_1). The para-substituted congener **83B** had worse characteristics with 3-fold reduced affinity at M_2 receptors (pK_i = 7.53), low selectivity (3-fold M_2/M_4) and lower specificity (68-fold M_2/H_1). Taken together, no clear relationship was observed for a preferred site of substitution concerning absolute affinity at M_2 as the pK_i value was increased (**78B** → **79B**), remained unchanged (**80B** → **81B**) or was diminished (**82B** → **83B**). Concerning

muscarinic receptor subtype selectivity, the meta-substituted derivatives showed better results. All compounds had sufficient specificity for M_2 versus H_1 receptors (at least 30-fold for **80B**).

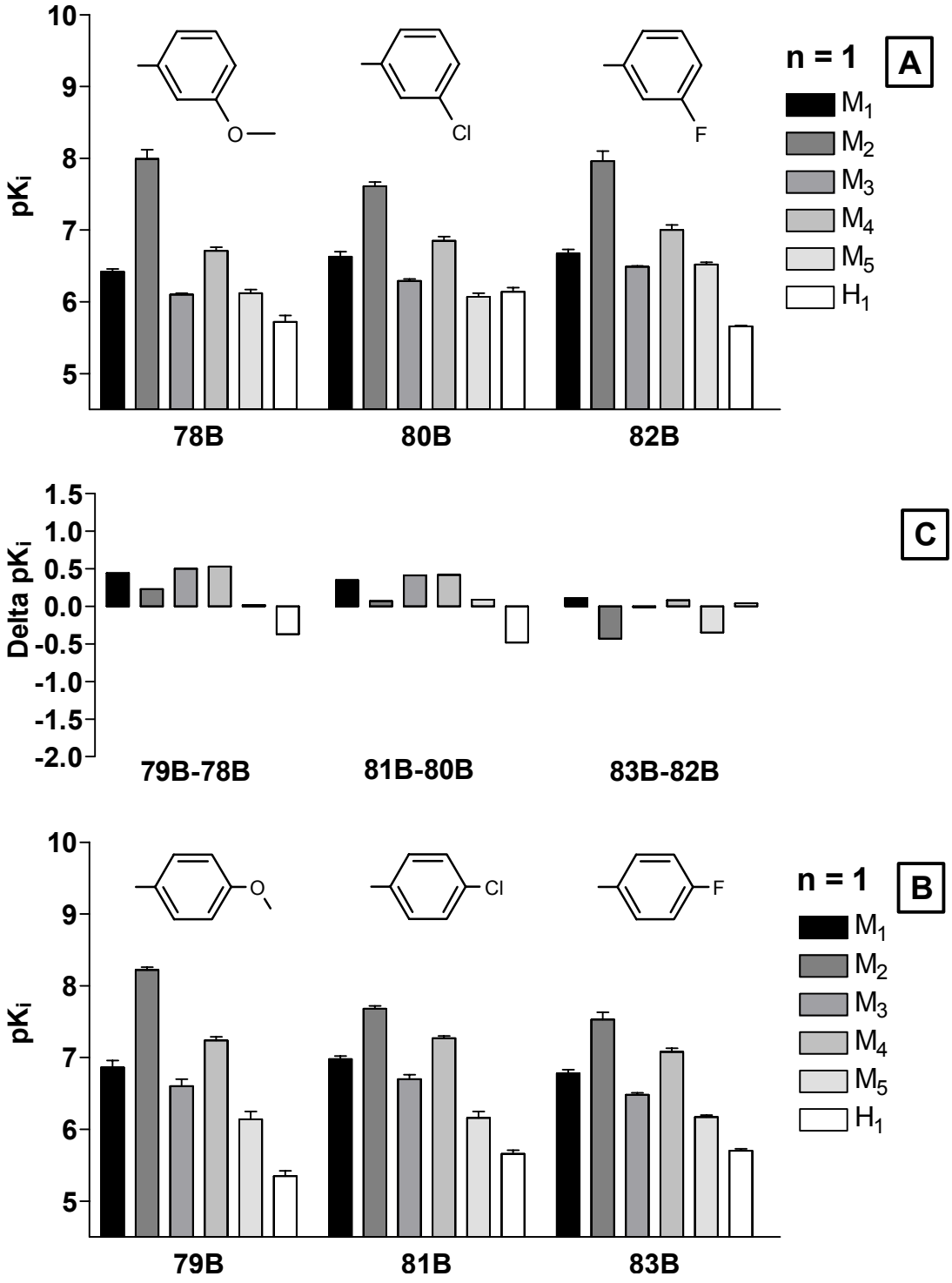


Fig. 4.43 *Affinity values (pK_i) of compounds related to **72B** with substituents in meta-position (**78B**, **80B**, **82B** (**A**)) or para-position (**79B**, **81B**, **83B** (**B**)) at the phenylethyl group and comparison of resulting changes in affinities (**C**) at $M_{1\text{-}5}$ and H_1 receptors.*

5 Discussion

5.1 General considerations

As shown for all used assays in the Results chapter (4.1), obtained K_D values for the various radioligands and pK_i values of reference drugs were in good agreement with literature data. Hill coefficients of competition curves were in most cases not significantly different from unity, consistent with a pure competitive inhibition according to a one-site binding model and the law of mass action.

5.2 Analogues of ondansetron at 5-HT_3 receptors

It was more than a decade ago when ondansetron (**1**) was characterised as an antiemetic drug in animal studies (Stables et al., 1987) and its pharmacological properties were described in various functional animal models at 5-HT_3 receptors (Butler et al., 1988). Ondansetron was launched to the market in 1990 by Glaxo, followed by granisetron in 1991 by Smith Kline Beecham and tropisetron by Sandoz in 1992 (Gaster and King, 1997). Since then more than 30 compounds were and still are under clinical research. Many clinical trials were stopped because no effective therapeutic benefit was found next to anti-emesis, providing enough cashflow to compensate development costs. With irritable bowel syndrom as a new therapeutic aim, research got more interesting lately. **1** was extensively used in SAR research to find new 5-HT_3 antagonists. This work led to alosetron which is closely related to **1** (Fig. 1.12). Currently, ramosetron, which also is a congener of **1,** is under clinical evaluation with affinity to 5-HT_3 receptors in picomolar range (Rabasseda, 2002). The introduction of 5-HT_3 antagonists can be considered as a mile stone in cancer therapy. With this new class of drugs new, more effective therapeutic options became possible in cancer therapy, which formerly were not tolerated in human patients due to severe side effects, especially massive vomiting and nausea. A good control of acute vomiting can be achieved using the 5-HT_3 antagonists actually approved. However, these drugs are less effective in prevention of delayed sickness. Ramosetron was recently shown to have prolonged efficacy in prevention of vomiting. The question of side effects was lately raised for several 5-HT_3 antagonists, which are in general believed to be a very save class of drugs (Keefe, 2002). As a matter of fact, latest analyses showed cardiac side effects (elongation of QT-interval)

to be more frequently in dolasetron therapy in comparison to granisetron and ondansetron. Ondansetron was reported to cause more CNS side effects (Goodin and Cunningham, 2002). As patents for the older drugs are running out in the next years and new therapeutic targets for 5-HT_3 antagonists are under research, new compounds are still of interest.

5.2.1 Influence of substitution pattern at the imidazole moiety

In SAR research done at Glaxo it was found that the unsubstituted indole congener of ondansetron (**1**) had strong interactions with CYP450 enzymes (Oxford et al., 1992). Introducing substituents at position 2 or 4 of the imidazole ring reduced this interaction and increased affinity to 5-HT_3 receptors. Smaller substituents were found to result in higher affinities in a series of indole congeners with an optimum for a methyl substituent in position 2 (→ ondansetron **1**). Incorporating the side chain of **1** into a tetrahydrocarbazolone system, led to increased oral activity. In our studies with analogues of **1** at 5-HT_{3A} receptors we found the derivative **2A** with a methyl group at position 4 to be slightly more potent than the parent compound with a methyl group in position 2 (pK_i = 9.05 and 8.83, respectively). This is in contrast to the findings with the indole congeners of Glaxo (Oxford et al., 1992), where findings were vice versa for the preferred substitution pattern (data at rat vagus nerve pA_2 = 8.0 and 7.4 for substituents in 2 and 4 position, respectively). Compounds with substituents at position 5 ("**B**"-series; Table 4.6) were less potent than analogues with substitution at position 4 ("**A**"-series) up to 51-fold for **4A/B**. Similar results were obtained in functional studies in guinea-pig ileum. With increasing size of substituent, affinity decreased exemplified for compounds **2A** - **6A** in Fig. 5.1 (**A**). It was very interesting to notice that the ratio of binding affinity at h5-HT_{3A} and guinea-pig ileum functional values decreased with increasing size of the substituent, shown in Fig. 5.1 (**B**). One can speculate that the substitution pattern at the imidazole moiety is more critical at h5-HT_{3A} than at gp-5HT_3 receptors, as bulky substituents were better tolerated in guinea-pig ileum. Similar findings were obtained for compounds **7** - **10** with substituents at position 2. Increasing size of the substituents decreased affinities in agreement with the findings at Glaxo (Oxford et al., 1992). Fig. 5.2 (**A**) gives the affinities of compounds **1** and **7** - **10** next to the ratios of binding data and functional data (**B**). The methyl group of the parent compound was an optimum as affinities

decreased 4-fold, 9-fold, 25-fold and > 21380-fold **1** → **7** - **10**, respectively. With increasing size of substituents ratios decreased, as seen already for compounds **2A/B** - **6A/B**.

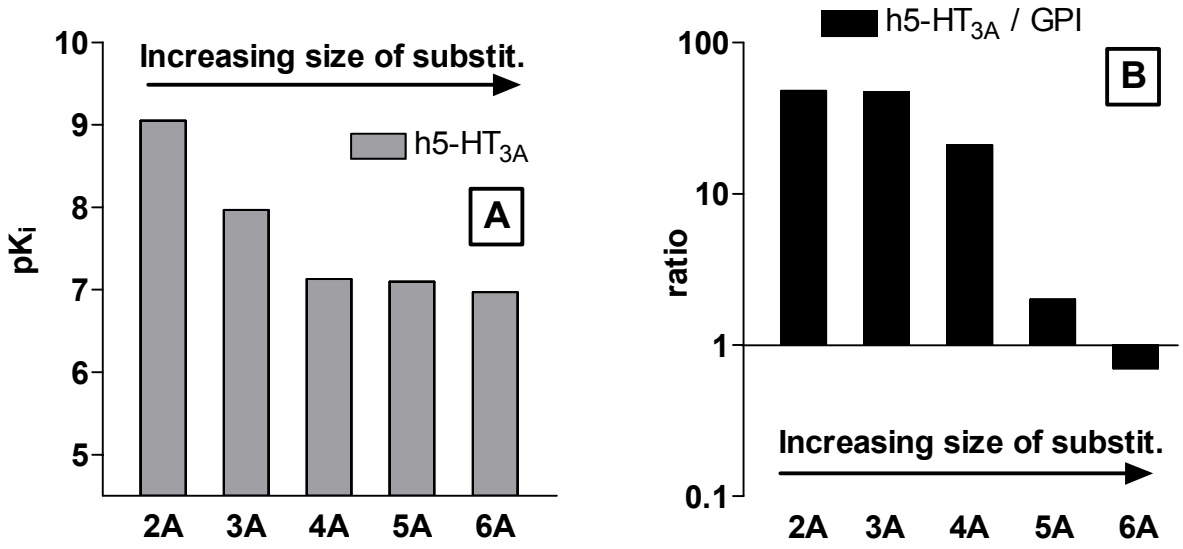


Fig. 5.1 *Affinities of compounds **2A** - **6A** with different substituents at position 4 of the imidazole ring at h-5HT$_{3A}$ (**A**) and ratios of binding and functional data (**B**).*

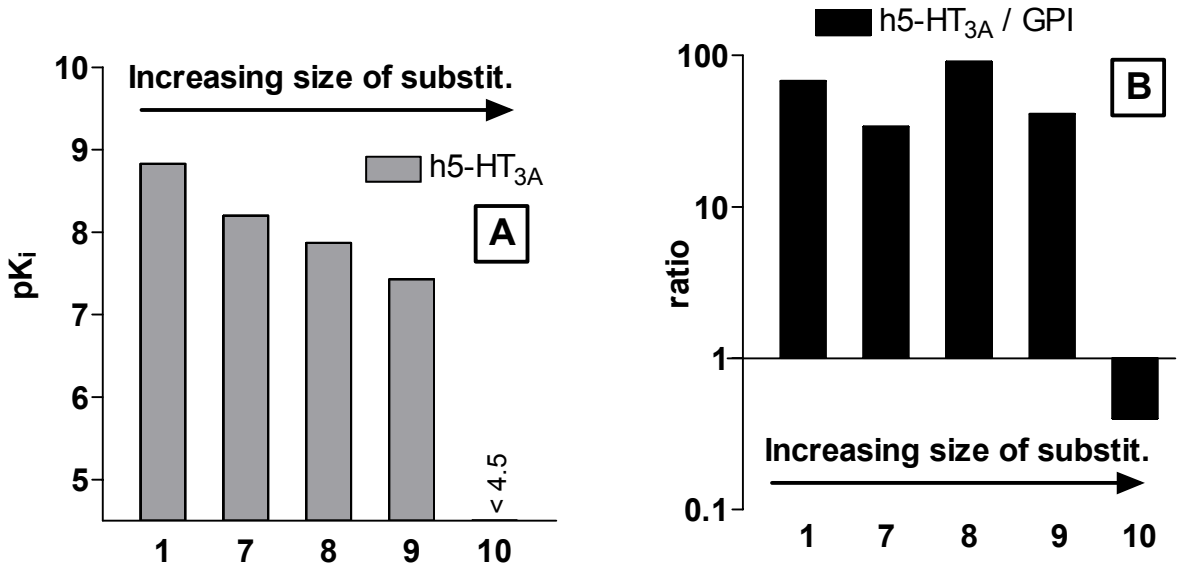


Fig. 5.2 *Affinities of compounds **1** and **7** - **10** with different substituents at position 2 of the imidazole ring at h-5HT$_{3A}$ (**A**) and ratios of binding and functional data (**B**).*

5.2.2 Effect of quaternization

N-Methylation of the unsubstituted imidazole ring at position 3 led to compound **11**. A 19-fold reduction in affinity was seen compared to **1**. Almost the same affinities were found for **1** and its N-methylated derivative in tests at rat vagus nerve carried out at Glaxo with pA_2 = 8.6 and 8.4 (Oxford et al., 1992), leading to the theory that the imidazole ring might be protonated in the binding interaction with the receptor. However, other studies showed different effects for N-methylation depending on the structure of the free base. Cases with no changes in affinities as well as 277-fold reduced affinities caused by N-methylation were reported (Cappelli et al., 2002).

5.2.3 Stereochemical aspects

In our series only one pair of enantiomers was tested **(+/-)-14** and a stereoselectivity ratio of 26 was calculated. The influence of stereochemistry seems to be strictly related to the structure of the molecule. Almost identical values were measured for the enantiomers of **1** in functional (Butler et al., 1988) and binding studies (Kilpatrick et al., 1987). In another study, there was no difference in affinity for some pairs of enantiomers and for other compounds stereoselectivity ratios of 100 were found depending on size and shape of the basic centre (Cappelli et al., 2002). In one study inverse stereoselectivity was found at 5-HT_3 and D_2 receptors and compounds with high specificity for 5-HT_3 and D_2 receptors were obtained by investigating pure enantiomers (Hirokawa et al., 2002).

5.2.4 Compounds related to ketanserin

Compounds **13** - **15** were hybrids of ketanserin, a highly potent reference 5-HT_2 antagonist, and ondansetron. Compounds **(+/-)-14** had the amino moiety of ketanserin and the tetrahydrocarbazolone moiety of ondansetron, whereas **13** lacked the substituent attached to the piperidine ring. Higher affinities at recombinant 5-HT_{3A} receptors were found for **13** compared to functional 5-HT_2 data at rat tail artery (pk_i = 8.16 and pA_2 = 5.98) (Elz and Heil, 1995). Interestingly, the complete ketanserin residue in **(+)-14** and **(-)-14** generated the inverse result (pK_i = 5.00, 6.41 and pA_2 = 7.19 and 9.36) with much higher affinities at 5-HT_2 receptors, showing this part of the

molecule to be of special interest to obtain compounds with high affinities to either 5-HT_2 or 5-HT_3 receptors. Further increase of the substituent in **15** led to total loss of affinity at 5-HT_{3A} in contrast to 5-HT_2 receptors.

5.2.5 Receptor diversity and species differences

In an early stage of research for 5-HT_3 antagonists, pronounced differences were found concerning the pharmacological properties of tested compounds in different functional animal models and binding studies. Some of the most prominent discrepancies were found in test at guinea-pig ileum in comparison to studies at rat vagus nerve (Butler et al., 1990), especially in agonist characteristics. Additionally, electrophysiological data revealed heterogeneity in different tissues with unequal electrophysiological properties of the measured single ion channels (Peters et al., 1992). A lot of speculation about different subtypes and species variations were proposed. Facts were not clarified before cloning techniques came up to solve these questions. First cloning of a 5-HT_3 receptor subunit was reported from NCB20 cells (mouse neuroblastoma x chinese hamster embryonic brain cells) (Maricq et al., 1991). Since than, several splice variants and additional subtypes were identified in several species.

5.2.5.1 5-HT_3 splice variants

For several laboratory animals 5-HT_3 receptors were cloned in the last 10 years, e.g. mouse (Hope et al., 1993; Werner et al., 1994), rat (Miquel et al., 1995), and finally guinea-pig (Lankiewicz et al., 1998). Two splice variants were identified differing in 5 or 6 amino acids, termed 5-HT_{3AS} (short) and 5-HT_{3AL} (long) for each species. The human 5-HT_3 receptor cDNA was isolated and showed 85% and 84% identity to mouse and rat, respectively (Miyake et al., 1995). Two additional splice variants were reported for the h5-HT_{3A} receptor (Brüss et al., 2000b), a truncated form 5-HT_{3AT} and a longer variant 5-HT_{3AL}. Splice variants of animals and humans do not correspond. This was clarified by mapping of the mouse and human 5-HT_{3A} gene structures (Werner et al., 1994; Brüss et al., 2000b). Pharmacological characterisation of the human splice variants expressed in HEK293 cells showed that non of the splice isoforms formed a functional channel alone but modified channel properties when

they were coexpressed with h5-HT_{3A}. Binding studies showed no differences for [^{3}H]GR65630 binding affinity at cells with additionally expressed splices variants. However, electrophysiological studies showed some parameters to be modified (Brüss et al., 2000a) when splice variants were coexpressed.

Taken together, splice variants of 5-HT_3 subunits were found in rodents and man, but they did not correspond to each other. Receptor diversity due to splice variants was only of minor relevance for pharmacological properties and not sufficient to explain former discrepancies (Fletcher and Barnes, 1998; Peters et al., 1992). As some findings were still not explainable, the search for further subunits continued. Good reasons for the existence of further subunits came from purification studies of pig 5-HT_3 receptors, finding other proteins attached to the receptor in addition to the 5-HT_{3A} subunit (Fletcher and Barnes, 1998).

5.2.5.2 5-HT_3 receptor subunits

Another 5-HT_3 receptor subunit, 5-HT_{3B}, was identified in human (Davies et al., 1999; Dubin et al., 1999), mouse and rat tissue (Hanna et al., 2000). Studies showed that the 5-HT_{3B} subunit was not able to assemble to functional channels alone but modified electrophysiological properties, when it was coexpressed with the 5-HT_{3A} subunit. Distribution studies in rat showed both subunits in the peripheral nervous system (PNS) but only the 5-HT_{3A} subunit in the CNS. In the PNS more neurons expressing the A than the B subunit were detected. 90% of cells containing the B subunit coexpressed the A subunit. In human tissues actually no direct prove for the abundance of the B subunit was shown. RT-PCR studies (Davies et al., 1999) and northern blot analysis detected the B subunit in several human tissues in CNS and PNS. However, PCR studies were shown to be critical, because the protein might not be produced even though PCR studies amplified the mRNA transcript (Stewart et al., 2003). Up to now, one can not say for sure whether 5-HT_{3B} is expressed in human brain (van Hooft and Yakel, 2003). With the identification of this second subunit some differences in channel conductance that were found between native neurons and cell lines (Hussy et al., 1994) could be explained (Hanna et al., 2000). In rodents and humans, heteromeric channels possess higher single channel conductances than homomeric channels. The question of physiological relevance remains still unclear, as in a lately published study the pharmacological profile for homomeric A and

heteromeric A/B channels was shown to be almost identical (Brady et al., 2001) and the proposal was made that rather the biophysical properties of the channels were changed. The relevance of assembly with subunits of other receptor families, e.g. subunits of nACh receptors (van Hooft and Yakel, 2003), needs further evaluation. Recently, h5-HT_{3C} (Dubin et al., 2001), h5-HT_{3D} and h5-HT_{3E} subunits were cloned and the gene structures were identified (Niesler et al., 2003). Comparison of the distribution pattern of all known subtypes with RT-PCR technique showed the A, B and C subunit to be widely expressed, whereas the D subunit was only found in kidney, colon and liver. mRNA of the E subunit was even more restricted and only detected in colon and small intestine (Niesler et al., 2003). It is very likely that heteromeric receptors are built and contribute to receptor heterogeneity in humans. Further studies are needed providing direct evidence for distribution of the described subtypes. The physiological role of receptor diversity remains to be elucidated, too.

5.2.6 Correlation of binding and functional data

We observed marked differences between binding data at h5-HT_{3A} and functional data at native gp5-HT_3 in guinea-pig ileum. Fig. 5.3 gives a comparison of affinity data for some reference drugs obtained in the most important animal models (RVN, GPI) and in binding studies at cloned human, rat and guinea-pig 5-HT_{3A} receptors. It can be clearly seen that binding data at rat and human receptors are identical to functional studies in rat vagus nerve. Functional data in guinea-pig ileum and binding affinity at cloned gp5-HT_3 receptors were comparable, too, whereas pronounced differences were seen in comparison to human and rat data.

In our series of compounds, no clear relationship was found between binding affinity to h5-HT_{3A} and functional data at native gp5-HT_3 receptors, as binding experiments resulted in higher, equal or lower affinities. Fig. 5.4 shows a correlation of binding and functional data. It can be concluded that data obtained in studies with gp5-HT_3 receptors are not suitable for the development of new drugs in humans (Lankiewicz et al., 1998) and are of little predictive power with regard to the relevance in human tissues. 5-HT_3 receptors of human and guinea-pig can be considered as true species orthologues.

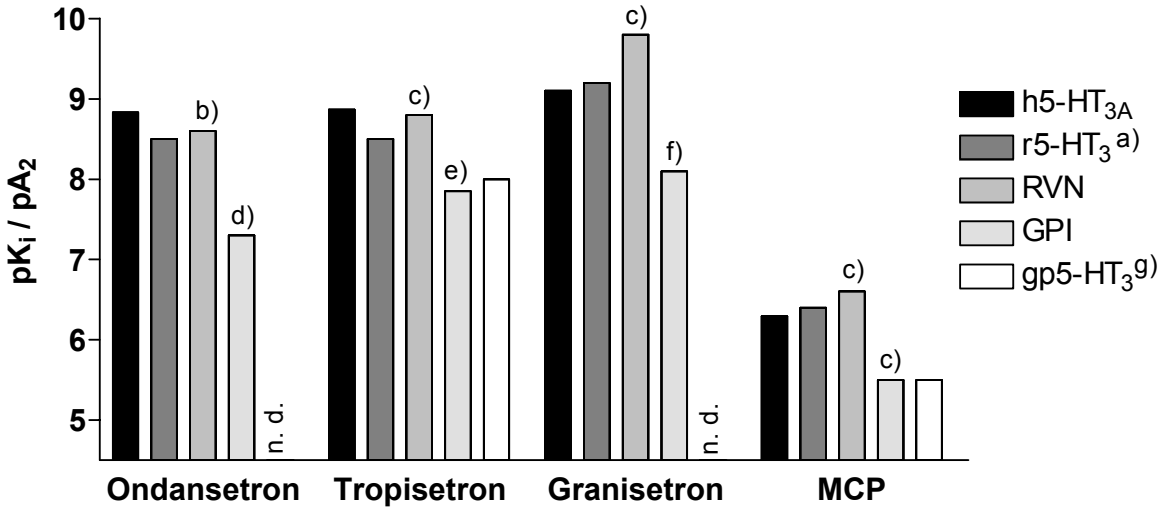


Fig. 5.3 *Comparison of affinities of some reference drugs in various 5-HT$_3$ models. Binding data at human, rat and guinea-pig receptors and functional data at rat vagus nerve (RVN) and guinea-pig ileum (GPI) are shown. [a)] Kilpatrick et al., 1987; [b)]Oxford et al., 1992; [c)]Kilpatrick et al., 1990a; [d)] Butler et al., 1988; [e)]Richardson et al., 1985; [f)] Sanger and Nelson, 1989; [g)] Lankiewicz et al., 1998. n.d. = not determined.*

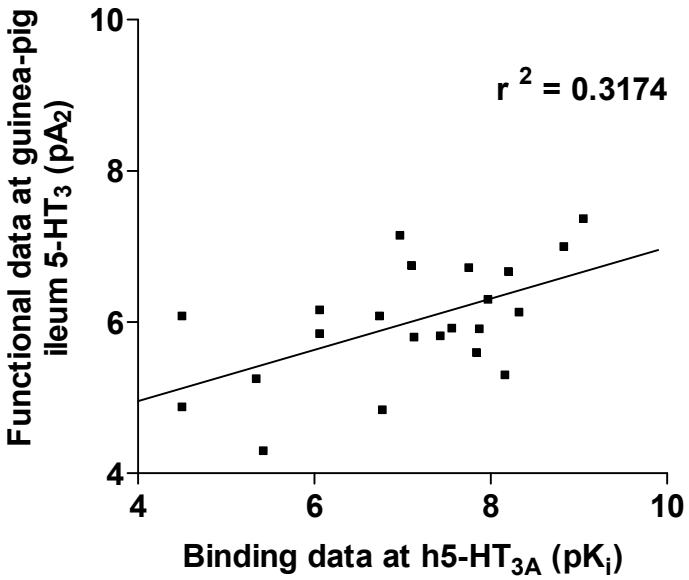


Fig. 5.4 *Correlation of binding data at h5-HT$_{3A}$ and functional data obtained in guinea-pig ileum for analogues of ondansetron. When affinities lay below detection level, the detection minimum was used as given in Table 4.6.*

5.3 Compounds related to metoclopramide at 5-HT_4 receptors

Metoclopramide as a starting point in research for prokinetic drugs had only low affinity to 5-HT_4 receptors (pK_i = 6.0 in binding studies in rat brain) and displayed partial agonism. Additional studies at 5-HT_3 and D_2 receptors revealed comparable affinities at these subtypes (pK_i = 6.4 and 6.5, respectively) (Yang et al., 1996). Many structural variations were carried out to find more potent derivatives with high specificity and selectivity in favour of the 5-HT_4 subtype. In 1991, the ester-congener of metoclopramide, SDZ205-557 (**19**), was shown to be more potent at 5-HT_4 receptors with pA_2 = 7.4 in guinea-pig ileum (Buchheit et al., 1991, 1992). However, only 3-fold selectivity was shown versus 5-HT_3 in binding studies (pK_i = 6.9) (Eglen et al., 1993a). With discovery of GR113808 as a highly potent and selective 5-HT_4 antagonist and radioactive labelling of this compound (Gale et al., 1994; Langlois et al., 1994) a new era started, using this newly available pharmacological tool for fast screenings (Grossman et al., 1993). In our study we evaluated the binding affinities of compounds related to **19** and compared the data with previously obtained data in functional 5-HT_4 models in guinea-pig ileum and rat oesophagus. Several studies on esters derived from their benzamide congeners were carried out (Elz and Keller, 1995; Yang et al., 1997). They confirmed the findings of metoclopramide and **19** that the esters had increased affinities at 5-HT_4 receptors. For the benzamide congener of the carboxylic acid ester ML10302 (**22**), an approximately 100-fold lower affinity was found (Yang et al., 1997).

5.3.1 Substitutions in the side chain

In our studies, the introduction of methyl groups in the side chain resulted in dramatically decreased affinities in compounds with acyclic amino moieties (**19** → **(±)-20** (56-fold), **19** → **(±)-21** (12-fold) and **22** → **(+)-23** (13-fold)). The position next to the ester group seems to be more critical for substitution, as the decrease was more pronounced in **(±)-20** in comparison to substitution at the next chain carbon atom at **(±)-21** and **(+)-23**. This is in good agreement with literature in studies with conformationally fixed substituents in the side chain (Yang et al., 1996).

5.3.2 Influence of the piperidine ring

Insertion of the basic nitrogen in a piperidine ring system (**19** → **22** (ML10302)) resulted in a 3-fold increase in affinity (pK_i = 7.83 and 8.37, respectively). The same affinity for **22** was found in other binding studies, too (Yang et al., 1997). This compound displayed good selectivity versus 5-HT_3 (pA_2 = 5.9 in guinea-pig ileum) and possessed partial agonism in 5-HT_4 receptor studies in rat oesophagus and guinea-pig ileum (Elz and Keller, 1995). Studies (Yang et al., 1997) showed that introduction of substituents at the piperidine ring system was tolerated. Most of these analogues were partial agonists. Interestingly, the 3, 5-dimethyl derivatives behaved as full antagonists. Introduction of a bulky substituent (**22** → **(±)-26**) was well tolerated (pK_i = 8.43). The congener missing the ketone oxygen of **(±)-26** was found to have comparable affinity of pK_i = 8.1 (Yang et al., 1997). Thus the ketone functionality was not relevant for binding.

5.3.3 Modification of chain length

We tested two series of compounds with modification of chain length (Fig. 4.13 and Fig. 4.14). Compounds **22**, **24** and **25** had an unsubstituted piperidine ring and elongated side chain length with 2, 3 and 4 carbon atoms. Additionally, compounds **27** - **29** with a benzyl substituted piperazine moiety were investigated. In the case of the analogues with a piperidine ring system elongation from 2 to 3 carbon atoms led to a decreased affinity (**22** → **24** (10-fold)). Elongation to 4 atoms (**24** → **25**) resulted in a further 8-fold decrease in affinity. Comparable decreases in affinity were seen in guinea-pig ileum (13-fold and 2-fold). Functional data for the 5-HT_4 antagonist **24** (RS23597-190) were in agreement with literature data (Eglen et al., 1993b). **24** was shown to be highly selective versus 5-HT_3 receptors in binding studies (pK_i = 5.7; Eglen et al., 1993b). Interestingly, findings were very different for the piperazine derivatives. Elongation of the side chain from 2 to 3 carbon atoms (**27** → **28**) resulted in a 2-fold increase in affinity. Further elongation to 4 atoms (**28** → **29**) did not induce significant changes in affinity. It was surprising to find the phenyl congener **30** to be 43-fold more potent than the benzyl analogue **27** (pK_i = 8.28 and 6.65). Comparable affinities for **30** were found in literature (Curtet et al., 2000). Our findings indicate that the influence of side chain length on binding affinity depends on the structure of the

amino moiety, as different results were obtained for piperidine and piperazine derivatives. Different lengths of the spacer between the hydrogen bond acceptor (the carbonyl carbon atom) and the basic nitrogen atom are tolerated: Spacer with four bonds (e.g. in **19**) up to 6 bonds (e.g. GR113808) resulted in potent compounds (Langlois and Fischmeister, 2003). In comparison to **30**, analogues with heteroaryl substituents (pyrimidine, pyrazine, pyridazine and pyridine) were shown to be more potent with pK_i values of approximately 9.0 (Curtet et al., 2000). Reduction of chain length to only one carbon atom was reported to result in decreased affinities in a set of pyrrolizidine analogues (Eglen, 1998).

5.3.4 Stereochemical aspects

We tested one pair of enantiomers (**(+/-)-23**) in this series. In functional studies, a stereoselectivity ratio of 2 was calculated, whereas a factor of 8 was calculated by using data obtained in binding experiments. For several other compounds related to metoclopramide low stereoselectivity was reported at 5-HT_4 receptors. This includes rencapride (no stereoselectivity) and zacopride (factor 4) (Yang et al., 1997) with a centre of chirality in the basic amino moiety. These results indicate this molecule part to be not critical for binding affinity at 5-HT_4 receptors. This hypothesis is supported as bulky substituents at the basic nitrogen moiety are tolerated and resulted in highly potent compounds (Langlois and Fischmeister, 2003). Taken together, the influence of stereochemistry depends strongly on the size and shape of the basic moiety.

5.3.5 Comparison of pharmacophores at 5-HT_3 and 5-HT_4 receptors

Both receptors share some general properties with regard of the pharmacophores of compounds of the benzoate type. An aromatic ring with a coplanar attached acceptor group for hydrogen bonding is necessary as well as a basic nitrogen moiety. At the 5-HT_4 receptor, an oxygen atom next to the hydrogen bond acceptor group is needed for most reported structures. A major difference was found for the size of the basic centre. Bulky substituents are not tolerated at the binding site of 5-HT_3 receptors, whereas 5-HT_4 receptors can fit voluminous groups. Usually benzoate compounds are antagonists at 5-HT_3 receptors. At 5-HT_4 receptors agonists, partial agonists and

antagonists were found. Minor changes were sometimes sufficient to convert a partial agonist into an antagonist.

5.3.6 Receptor diversity and distribution pattern

Cloning techniques brought up several splice variants of the 5-HT_4 receptor. In the following chapter the current knowledge is summarised and the implications for receptor diversity are discussed.

5.3.6.1 Splice variants

For h5-HT_4 receptors several splice variants were cloned and characterised: h5-$HT_{4(a)}$ (Blondel et al., 1997; Claeysen et al., 1997), h5-$HT_{4(b)}$ (Van den Wyngaert et al., 1997), h5-$HT_{4(c)}$ (Blondel et al., 1998), h5-$HT_{4(d)}$ (Blondel et al., 1998; Mialet et al., 2000b) and h5-$HT_{4(e)}$ (Mialet et al., 2000a), which was later renamed h5-$HT_{4(g)}$ when the gene structure was discovered (Bender et al., 2000). All these splice variants are identical up to amino acid 358 and differ only in the intracellular C-terminus. Lately, another short splice variant lacking the C-terminus was cloned and named h5-$HT_{4(n)}$ because “non” of the known C-termini is expressed (Vilaro et al., 2002). In contrast to these splicing sites one splice variant resulting in an insertion in the second extracellular loop named h5-$HT_{4(h)}$ was reported, which can be combined with all the other splice variants at the C-terminus (Bender et al., 2000). This splice variant was cloned together with the C-terminus of h5-$HT_{4(b)}$ resulting in h5-$HT_{4(hb)}$. With regard to the h5-HT_4 receptor gene structure, eight splice variants, h5-$HT_{4(a-g)}$ and h5-$HT_{4(n)}$, concerning the C-terminus were found and one additional splicing site at the extracellular domain 2, named h5-$HT_{4(h)}$, resulting in 16 possible splice variants. For rat, r5-$HT_{4(a)}$, r5-$HT_{4(b)}$ (Gerald et al., 1995) and r5-$HT_{4(e)}$ (Claeysen et al., 1999) were cloned. For mouse, m5-$HT_{4(a)}$, m5-$HT_{4(b)}$ (Claeysen et al., 1996), m5-$HT_{4(e)}$ and m5-$HT_{4(f)}$ were cloned (Claeysen et al., 1999).

All splice variants display similar pharmacological profiles concerning binding affinities of most compounds (Curtet et al., 2000; Langlois and Fischmeister, 2003). This is not surprising, as most splice variants display exactly the same membrane topology differing only at the intracellular C-terminus. Differences were found for agonist potencies and constitutive activity. At h5-$HT_{4(d)}$ the reference antagonist

RS39604 behaved as a partial agonist and rencapride acted as full agonist instead of a partial agonist (Mialet et al., 2000b). For h5-$HT_{4(n)}$ (Vilaro et al., 2002) and h5-$HT_{4(e)}$, now termed h5-$HT_{4(g)}$ (Mialet et al., 2000a), the reference antagonist GR113808 was found to be an inverse agonist. At h5-$HT_{4(hb)}$ GR113808 was shown to possess partial agonism (Bender et al., 2000). The physiological relevance of these findings is doubtful as different cell lines were used and direct comparability is not possible. Further research showed some physiological implications of the different splice variants. It was shown that h-5$HT_{4(b)}$, but not h-5$HT_{4(a)}$, had the possibility to interact with $G_{i/o}$ in addition to the well known interaction with G_s (Pindon et al., 2002). Studies showed that alternative splicing of the C-terminus results in modulation of constitutive activity. Regulation of basal activity was shown to be influenced by agonist-dependent receptor palmitoylation (Ponimaskin et al., 2002). In addition, an important role concerning receptor desensitisation was found for the C-terminus and influence of PK-A was shown (Mialet et al., 2003). It is likely that receptor fine tuning is regulated by different expression of splice variants, resulting in subtle modifications of basal activity and desensitisation characteristics.

5.3.6.2 Tissue distribution

Several studies were performed in the hope to find discrete expression for some splice variants of the 5-HT_4 receptor in the CNS, peripheral nervous system (PNS) or a single organ. Hopes were disappointed when several studies showed a very complex expression pattern of subtypes with no subtype being exclusively expressed in a single region. On the other hand no tissue was found expressing only one subtype (Vilaro et al., 2002; Bach et al., 2001; Medhurst et al., 2001). It is important to note that all these studies were RT-PCR studies. Up to now no direct evidence concerning the expression pattern of the single subtypes is available. One study reported a quantitative mRNA analysis in several tissues for five splice isoforms (Medhurst et al., 2001). From mRNA analysis it was concluded that the most abundant receptor is h5-$HT_{4(b)}$. This mRNA subtype was widely distributed in CNS and PNS. High mRNA levels were found in various regions of the CNS for the a, b, c and g splice variants. The h5-$HT_{4(d)}$ subtype was only found in the small intestine at low levels. This study provided indirect evidence for expression of 5-HT_4 receptors at

higher levels in small intestine and uterus. Lower levels of mRNA were found in heart, testis, stomach and other tissues.

5.3.7 Correlation of binding and functional data

With the cloning of 5-HT_4 receptors from rodents direct comparison to human receptors became possible. No pharmacological differences were seen between r5-$HT_{4(a)}$ and h5-$HT_{4(a)}$ receptors. Similar findings were reported for binding studies at h5-$HT_{4(b)}$, guinea-pig brain preparation and a subtype of rat (Van den Wyngaert et al., 1997). Data obtained in functional 5-HT_4 studies in guinea-pig ileum and rat oesophagus showed very good correlation (Elz and Keller, 1995). Fig. 5.5 gives a comparison of binding studies at h5-$HT_{4(b)}$ and guinea-pig brain and functional data at guinea-pig ileum and rat oesophagus for some reference compounds. Very good correlation can be seen for functional and binding data obtained at human, rat and guinea-pig receptors.

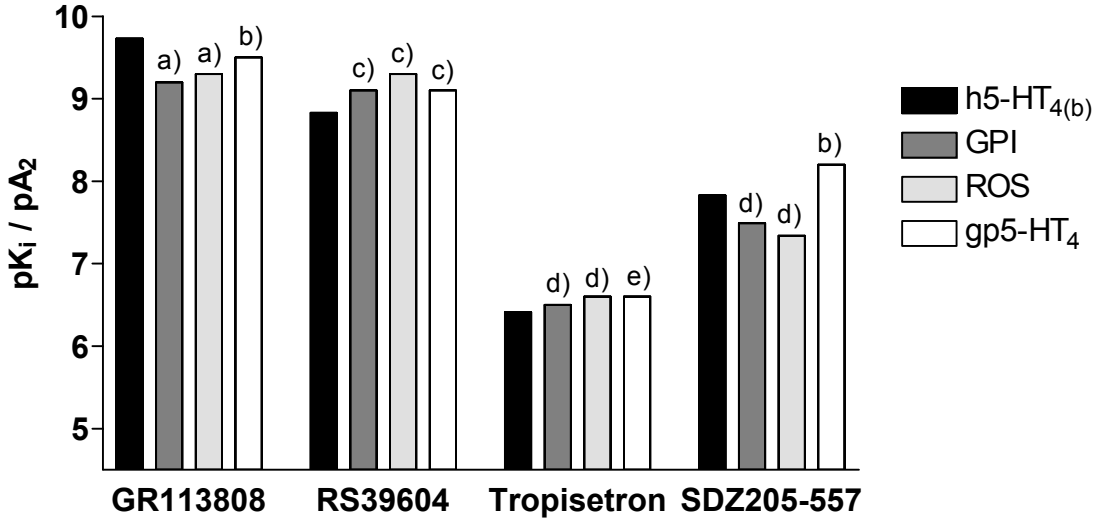


Fig. 5.5 *Comparison of affinities of some reference drugs in various 5-HT_4 models. Binding data at human and guinea-pig and functional data at guinea-pig ileum (GPI) and rat oesophagus (ROS) are shown.* [a] *Gale et al., 1994 - functional data were obtained in guinea-pig colon;* [b] *Grossman et al., 1993;* [c] *Hegde et al., 1995;* [d] *Elz and Keller, 1995;* [e] *Van den Wyngaert et al., 1997.*

Fig. 5.6 gives a correlation of binding data at h5-$HT_{4(b)}$ with functional data obtained in guinea-pig ileum. In contrast to 5-HT_3 receptors (Fig. 5.4) a good correlation was seen between human and guinea-pig data. It can be concluded that 5-HT_4 receptors of human, guinea-pig and rat are true species homologues and data obtained in one of these species, as well in binding studies as in functional studies, are of good predictive power for relevance in human clinical studies.

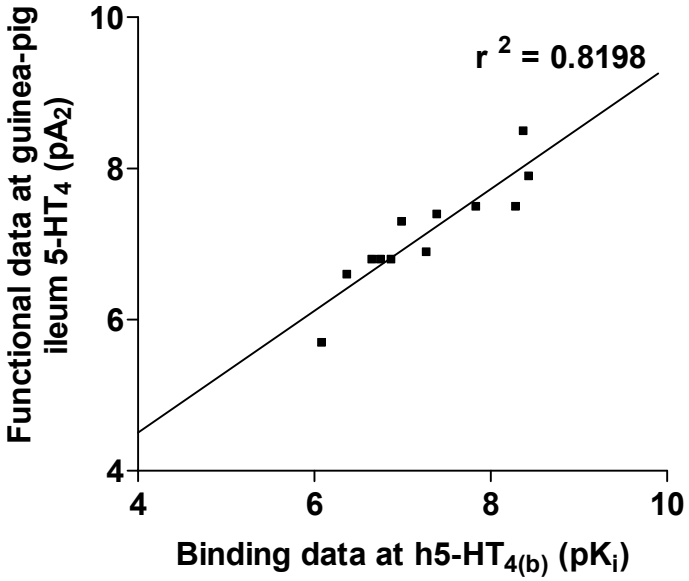


Fig. 5.6 *Correlation of binding data obtained at h5-$HT_{4(b)}$ and functional data obtained in guinea-pig ileum (GPI) for analogues of metoclopramide. Values for* ***25*** *were omitted as the compound was not completely dissolved.*

5.3.8 Therapeutic implications

Only tegaserod has reached the marked in the last years as a compound with partial agonism at 5-HT_4 receptors for use in C-IBS. Studies on IBS led to a more precise understanding of the role of 5-HT receptors in human GIT. 5-HT_3 and 5-HT_4 receptors were found on excitatory cholinergic neurons in the plexus myentericus. In addition, 5-HT_4 receptors are located on inhibitory nitrergic neurons (Camilleri, 2001). Thus, serotonergic receptors are an interesting therapeutic target for therapy of gastrointestinal disorders, especially in IBS. In the last years an increasing body of evidence for the influence of 5-HT_4 receptors in memory and cognitive performance came up. It was shown that 5-HT_4 receptors are involved in cholinergic transmission

in the brain (Matsumoto et al., 2001) and enhance the release of non-amyloidogenic sAPPα in CHO cells. Another report described 5-HT_4 receptor density to be unchanged in cortex of AD patients (Lai et al., 2003). Good results were obtained in reversal of a scopolamine-induced cognitive deficit in rats with partial 5-HT_4 agonists (Moser et al., 2002; Lelong et al., 2003). It is also noteworthy, that the combination of two per se ineffective doses of an AChE inhibitor and a 5-HT_4 partial agonist resulted in enhanced cognitive performance in rats (Lamirault et al., 2003). This might be a new therapeutic approach in cognitive disorders with reduced side effects due to lower doses of the administered drugs. Further use for 5-HT_4 partial agonists may be in voiding disorders associated with detrusor hypocontractility (Tonini and Candura, 1996). 5-HT_4 antagonists may be of interest as antiarrhythmic drugs inhibiting atrial flutter and fibrillation (Kaumann, 1994).

Actually no information is available concerning the specific role of different isoforms of 5-HT_4 receptors and their role in pathological conditions. Further direct prove for the distribution of the isoforms is needed. It will be a major future task to develop new compounds with selectivity for a single splice variant.

5.4 Analogues of McN-A-343

McN-A-343 (**31**) and its derivatives were extensively investigated in SAR studies at muscarinic receptor subtypes (Lambrecht et al., 1986, 1993, 1995; Moser et al., 1993, 1995; Keim, 2000) since first reports about **31** were given to act as a muscarinic ganglionic stimulant (Roszkowski, 1961). Studies showed **31** to be a functional selective agonist at muscarinic M_1 receptors with antagonistic or low partial agonist properties at M_2 and M_3 subtypes (Lambrecht et al., 1993). Insertion of the basic nitrogen into a pyrrolidine ring system, introducing a C1-methyl substituent and changes in substitution pattern at the aromatic ring resulted in increased affinity at M_1 receptors (Lambrecht et al., 1995). Increasing the substituent at C1 via ethyl to phenyl resulted in increased affinities at M_1. However, these compounds were no longer agonists but full antagonists.

In comparison to the effects at muscarinic receptor subtypes only little is known about SAR at other receptors such as 5-HT_3, 5-HT_4 and H_1. **31** was reported to be a weak antagonist at 5-HT_3 and 5-HT_4 receptors in the range of its affinity at muscarinic receptor subtypes (Sagrada et al., 1994). In our study we investigated several analogues of **31** at 5-HT_3, 5-HT_4 and H_1 receptors.

5.4.1 Studies at 5-HT_3 receptors

In functional studies at rat cervical ganglion and in binding studies with NG108-15 cells, low affinities for **31** were reported at 5-HT_3 receptors (pA_2 = 4.9, pK_i = 5.3). Affinities in binding experiments at M_1, M_2 and M_3 in tissue preparations were found to be in the same range with pK_i = 5.3, 5.2, 4.9, respectively (Sagrada et al., 1994). Our studies confirmed these findings at h5-HT_{3A} receptors (pK_i = 4.96). In our screening experiments many compounds were tested in one point screening experiments with a concentration of the cold competitor of 10^{-5} M. When no noteworthy inhibition was found (inhibition of specific binding < 40%, what is comparable to a pK_i of approximately 5.0) no complete competition curves were recorded to reduce costs.

5.4.1.1 Modifications at the aromatic ring

Changing the substitution pattern from m-Cl into p-F (**31** → **32**, **32**$^+$) led to reduced affinities (16% and 10% inhibition, respectively). This is in contrast to muscarinic receptor subtypes where p-substitution with several halogens was shown to increase affinities (Moser et al., 1993). Further more, compounds with acyclic amino moieties and a phenyl substituent at C1 were tested. **(R)-33**$^+$ with no substituent at the aromatic ring was found to be the most potent compound in this series (pK_i = 5.74) in contrast to its enantiomer (10% inhibition) and the related tertiary congeners (almost no inhibition). Introduction of an o-phenyl ring (**(R/S)-33**, **(R/S)-33**$^+$ → **(±)-34**, **(±)-34**$^+$) resulted in comparable affinities. **(±)-34**$^+$ was equipotent in comparison to the eutomer **(R)-33**$^+$ (pK_i = 5.58 and 5.74, respectively), whereas **(±)-34** had no detectable affinity. Insertion of the basic nitrogen in a pyrrolidine ring system and increase of the aromatic ring to a naphthyl moiety in **(±)-40**, **(±)-40**$^+$ led to affinities comparable to that of the parent compound (pK_i = 4.90 and 4.81, respectively). Replacing the aromatic ring with a diphenyl-methyl group (**(±)-41**, **(±)-41**$^+$) showed a 3-fold increase in affinity for the tertiary compound (pK_i = 5.45) and a slightly decreased affinity for the quaternary congener (32% inhibition). These findings at 5-HT_3 receptors are in contrast to those reported for muscarinic receptor subtypes, where introduction of bulky aromatic substituents resulted in pronounced losses in affinity (Keim, 2000 and K. Kreutzmann, personal communications). At the 5-HT_3 subtype, bulky substituents are tolerated in this position without pronounced losses in affinity. The exchange of a hydrogen into a fluorine atom (**(R/S)-37**, **(R/S)-37**$^+$ → **(R/S)-36**, **(R/S)-36**$^+$) was found to be without effect on binding affinities (inhibition for all compounds between 18 - 32%).

5.4.1.2 Compounds with a pyrrolidine ring and C1-substituents

A series of compounds with the basic nitrogen inserted into a pyrrolidine ring system were evaluated. Compounds had C1-substituents of increasing size and a 4-F substitution at the aromatic ring. No change in binding affinities at 5-HT_{3A} receptors could be seen for different C1-substituents in compounds **(R/S)-35**, **(R/S)-35**$^+$, **(R/S)-36**, **(R/S)-36**$^+$, **(R/S)-37**, **(R/S)-37**$^+$ (methyl, phenyl and 4-F-phenyl substituents, respectively). Non of these compounds inhibited more than 32% of specific binding. A 2-fold increase in affinity was measured for **(±)-39** and **(±)-39**$^+$ (pK_i = 5.25 and 5.22)

with a bulky 1-naphthyl substituent attached to the side chain. This clearly demonstrates this molecule part to be of little importance for 5-HT_3 affinity in contrast to muscarinic receptor subtypes, where dramatic changes were observed through modification of the C1-substituent (Keim, 2000 and K. Kreuzmann, personal communication). A direct comparison of the compounds **(R/S)-33**, **(R/S)-33$^+$** with an acyclic amino moiety with its pyrrolidine congeners, **(R/S)-37** and **(R/S)-37$^+$**, showed a decrease of approximately one order of magnitude in affinity compared to the quaternary eutomer **(R)-33$^+$**. All compounds with a pyrrolidine ring were less potent than the parent compound **31** unless a bulky substituent was introduced at C1, in **(±)-39** and **(±)-39$^+$**, or as the aromatic system at the carbamate nitrogen (**(±)-40**, **(±)-40$^+$** and **(±)-41$^+$**).

5.4.1.3 Ester analogues

Compounds **(±)-42** and **(±)-42$^+$** showed slightly higher affinities than their carbamate congeners **(±)-36** and **(±)-36$^+$**. Affinity of **(±)-42** was comparable to that of the parent compound **31** (pK_i = 4.96 and 5.08, respectively). Thus, exchange of the carbamate moiety into a carboxylic acid ester function was of little influence at 5-HT_3 receptors. This is in contrast to findings at muscarinic receptor subtypes, where affinity was drastically reduced (Keim, 2000).

5.4.1.4 Effects of quaternization

No clear relationship concerning the effect of N-methylation was found at 5-HT_3 receptors. Quaternary compounds possess higher, equal or lower affinities compared to their tertiary congeners. **(R)-33$^+$** and **(±)-34$^+$** showed higher affinities than the tertiary congeners (> 17-fold and > 12-fold). Comparable affinities were found for most tertiary and quaternary molecules with exception of **(±)-41**, where the tertiary compound was more potent. No clear structural relationship was seen. At muscarinic receptor subtypes some quaternary analogues were reported to be more potent than their tertiary congeners (Lambrecht et al., 1993, Moser et al., 1995). As ACh, the endogenous ligand, is a permanently charged molecule this is not very surprising.

5.4.1.5 Stereochemical aspects

Within the examined compounds several were tested as pure enantiomers. Most compounds showed little or no affinity to 5-HT_3 receptors. Only in the case of **(R)-33$^+$** a stereoselectivity ratio of approximately 10 was found, whereas no stereoselectivity was found for the other compounds.

5.4.2 Studies at 5-HT_4 receptors

Binding studies in pig brain tissue preparations and functional studies in rat oesophagus revealed considerable affinity of McN-A-343 (**31**) at 5-HT_4 receptors (pK_i = 5.9 and pA_2 = 6.2) (Sagrada et al., 1994). We were able to confirm these results in binding studies at h5-$HT_{4(b)}$ receptors (pK_i = 5.73). All tested analogues showed lower affinity at 5-HT_4 receptors compared to the parent compound.

5.4.2.1 Modifications of McN-A-343

Exchanging the m-Cl into a p-F substituent (**31** → **32**) resulted in a 10-fold decrease in affinity (pK_i = 5.73 and 4.70, respectively). This decrease was more pronounced than at h5-HT_{3A} receptors. Removal of the fluorine substituent and attachment of a C1-phenyl-substituent (**32** → **(R/S)-33** and **(R/S)-33$^+$**) did not result in a change in affinity in contrast to 5-HT_{3A} receptors. Insertion of the basic nitrogen atom into a pyrrolidine ring and adding a C1-methyl-substituent (**32** → **(R/S)-35** and **(R/S)-35$^+$**) resulted in slightly increased affinities. Exchanging the C1-methyl-substituent into a phenyl ring (**(R/S)-35**, **(R/S)-35$^+$** → **(R/S)-36**, **(R/S)-36$^+$**) was tolerated without changes in affinity. It is concluded that a C1-substituent is not critical for binding at 5-$HT_{4(b)}$ what is in good agreement with our findings at 5-HT_{3A} receptors. It is likely that the pyrrolidine ring is responsible for the observed small increase in affinity at 5-$HT_{4(b)}$. This is in contrast to our findings at 5-HT_{3A} receptors, where most pyrrolidine compounds possess lower affinity compared to the congengers with acyclic amino moieties. However, this is in good agreement with our findings with analogues of metoclopramide at 5-HT_4 receptors (5.3.2). As no tested derivative reached the affinity of the parent compound **31** it is concluded that the substitution pattern at the aromatic ring is the most critical part of the molecule concerning 5-HT_4 affinity.

5.4.2.2 Stereochemical aspects and influence of quaternization

As already mentioned in the results chapter, only a very weak tendency could be observed concerning stereoselectivity in favour to the (R)-configurated enantiomers. No clear tendency to higher binding affinities for tertiary or quaternary compounds was found for the charged or the uncharged molecules.

5.4.2.3 Proposal of a new 5-HT_4 ligand related to McN-A-343

Literature reports highly potent 5-HT_4 antagonists with phenylcarbamate structures (Eglen, 1998). These molecules have substituents in ortho-position of the aromatic ring as an important structural feature (Soulier et al., 1997). SAR work on phenylcarbamate analogues of metoclopramide showed compounds with piperidine ring systems to be most potent. A substituent in position 2 of the aromatic ring is necessary, whereas substituents in position 4 and 5 were dispensible in two SAR studies (Soulier et al., 1997; Oxford, 1993). Based on our findings and on literature data we propose a hybrid molecule consisting of either parts of metoclopramide and McN-A-343 as a new lead structure. Attachment of a methoxy substituent in position 2 might result in more potent compounds. Hybrid molecules consisting of a modified aromatic part of metoclopramide and the carbamate moiety attached to a butyne spacer of **31** (Fig. 5.7) might be a very unusual molecular structures for drugs with affinity at 5-HT_3 and 5-HT_4 receptors. For the basic amino group, a cis-3,5-dimethyl-piperidinyl ring was shown to be an optimum in other series of phenylcarbamates (Soulier et al., 1997). Substituents next to the ester group were reducing binding affinities in our series of metoclopramide analogues. Hence a hybrid molecule without a C1-substituent is most likely to be a potent 5-HT_4 ligand.

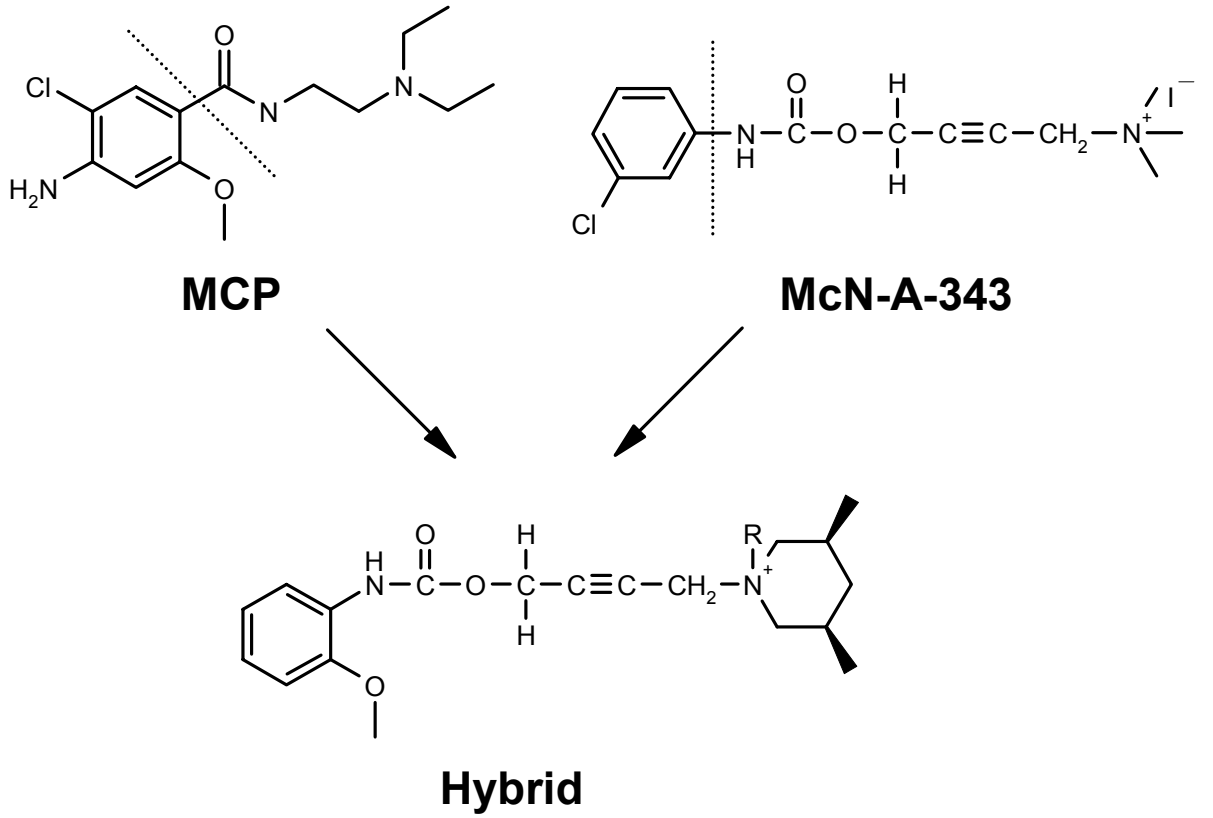


Fig. 5.7 *Proposal of a new class of 5-HT$_4$ ligands based on the molecular structures of metoclopramide (MCP) and McN-A-343.*

5.4.3 Studies at H_1 receptors

Compounds acting as antagonists at H_1 receptors are known for a long time. Up to now many new drugs were developed making the class of H_1 antagonists a complex mixture of chemical structures. However, analogues of McN-A-343 show a very unusual chemical structure for compounds of this pharmacological class and were therefore interesting to examine.

5.4.3.1 Influence of the amino moiety

For all tested compounds with acyclic amino moieties only low affinities were found with the highest values obtained for **(R)-33** (pK_i = 5.43). As shown in the results chapter, the direct comparison of compounds **33** and **37**, differing only in the amino moiety, showed pronounced higher affinities for the congener with a pyrrolidine ring

system (**37**). Size and shape of the amino moiety was an important feature for affinity to H_1 receptors.

5.4.3.2 Influence of C1-substituents

Modifications of size of the C1-substituent lead to a noticeable change in affinity. The optimum was found for a 4-F-phenyl substituent in **(±)-38** (pK_i = 6.98). Bulkier or smaller substituents led to lower affinities in comparison to **(±)-38**. Size and shape of the C1-substituent was a second important feature affecting affinity to H_1 receptors in this series.

5.4.3.3 Influence of the aromatic ring systems

Several modifications concerning the aromatic ring were introduced in **31**. Changing the substitution pattern from m-Cl to p-F (**31** → **32**, **32**$^+$) led to no change in affinity. Both drugs had no detectable affinity to H_1 receptors. Concerning the C1-phenyl substituted analogues one should notice that introduction of a comparable bulky o-phenyl substituent at the aromatic ring (**(±)-34**) resulted in an only 5-fold decrease in affinity in comparison to the unsubstituted eutomer **(R)-33** (pKi = 4.75 and 5.43, respectively). A comparable 5-fold decrease in affinity was found for the naphthyl analogue **(±)-40** in comparison to its phenyl congener eutomer **(R)-37** (pK_i = 6.12 and 6.72, respectively). It is noteworthy that the analogue **(±)-41** with a diphenyl-methyl group as an aromatic residue displayed unchanged affinity (pK_i = 6.74). Size and shape of the aromatic ring was less critical for H_1 affinity in our series of compounds compared to the amino moiety and the C1-substituent. Major changes at this part of the molecules resulted in only small changes in affinity.

5.4.3.4 Ester analogues and quaternization

Exchange of the carbamate moiety into an ester group (**36** → **42**) resulted in unchanged affinities. Thus, it is concluded that neither the carbamate structure was essential for H_1 affinity in our series nor was this a critical part in the molecule concerning interactions with the H_1 receptor. For all tested compounds, tertiary compounds displayed higher affinity values in comparison to their N-methylated,

quaternary congeners. This effect ranged from a 2-fold (in the case of **(±)-34** and **(±)-40**) to a maximum of 15-fold (found for **(S)-37** and **(S)-37$^+$**) higher affinities in favour to the tertiary compounds.

5.4.3.5 Stereochemical aspects

The (R)-configurated analogues were more potent at H_1 receptors than their (S)-configurated isomers. This was most prominent for **(R/S)-36$^+$** and **(R/S)-37$^+$** with a stereoselectivity ratio of factor 35. Previous binding studies at muscarinic receptor subtypes carried out in our laboratory by Dr. C. Keim (2000) had shown the inverse situation at mACh receptors. The (S)-configurated compounds were more potent than their enantiomers at $M_{1\text{-}5}$ receptors. For the enantiomers of analogues **33**, **33$^+$**, **36**, **36$^+$**, **37** and **37$^+$** a direct comparison was done between H_1 and M_1 affinities and stereoselectivity ratios were calculated (Table 5.1). High binding affinities, up to picomolar range (pK_i = 9.4), were measured at M_1 receptors (Keim, 2000). Fig. 5.8 gives the affinities for the stereoisomers of **33** and **33$^+$** at H_1 and M_1 receptors. Inverse stereoselectivity concerning H_1 and M_1 receptors can be seen at **33** and **33$^+$**. The (R)-configurated enantiomers were the eutomers at H_1 receptors, whereas the (S)-configurated isomers were the eutomers at M_1 receptors.

Table 5.1 *Stereoselectivity ratios for binding data of analogues of **31** at M_1 and H_1 receptors. These values are the antilogs of the differences between respective pK_i values (pK_i-(S) minus pK_i-(R)).*

Compound	stereoselectivity ratio M_1	stereoselectivity ratio H_1
(S) / (R)-33	85	0.3
(S) / (R)-33$^+$	59	< 0.5
(S) / (R)-36	158	0.1
(S) / (R)-36$^+$	138	0.03
(S) / (R)-37	1660	0.09
(S) / (R)-37$^+$	78	< 0.03

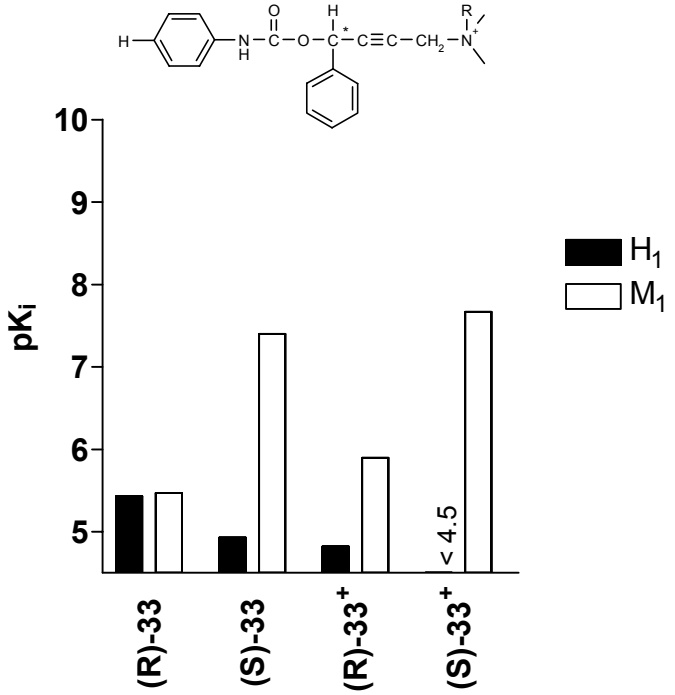


Fig. 5.8 *Binding affinities of the stereoisomers of **33** and **33**$^+$ at H_1 and M_1 receptors. M_1 binding data were measured by Dr. C. Keim in our laboratory (Keim, 2000).*

In Fig. 5.9 (**A**) and (**B**) the binding affinities of the closely related compounds **36** and **37** are shown. Comparison of **36** and **37** showed the F → H exchange to be bioisoster at M_1 and H_1 subtypes as binding data were almost identical. Inverse stereoselectivity concerning H_1 and M_1 receptors can be seen for **36**, **36**$^+$, **37** and **37**$^+$, but even more pronounced in comparison to **33** and **33**$^+$. The (R)-configurated enantiomers were the eutomers at H_1 receptors, whereas the (S)-configurated isomers were the eutomers at M_1 receptors. It is interesting to note, that **(S)-37**, which is the eutomer at M_1 receptors, is a highly potent M_1 antagonist with 5248-fold specificity versus the H_1 subtype, whereas **(R)-37**, which is the eutomer at H_1 receptors, is a H_1 antagonist with 3-fold specificity versus the M_1 subtype.

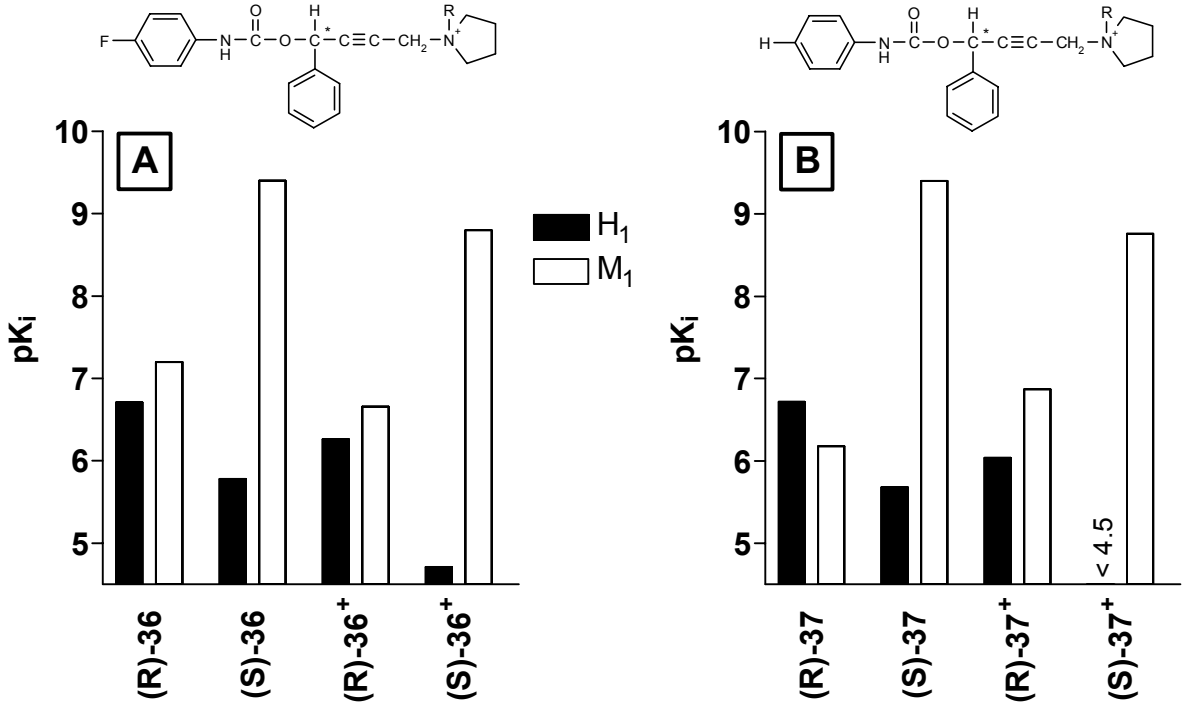


Fig. 5.9 *Binding affinities of the stereoisomers of **36**, **36**$^+$ (**A**) and **37**, **37**$^+$ (**B**) at H_1 and M_1 receptors. M_1 binding data were measured by Dr. C. Keim in our laboratory (Keim, 2000).*

Inverse stereoselectivity concerning H_1 and M_1 receptors has been reported in literature for several other compounds. In a series of compounds related to the H_1 antagonist ebastine, inverse stereoselectivity was shown, although to a much lesser extend as in our series (Zhang et al., 1994). Comparable findings were accounted for 3-methoxy-cyproheptadine in *in vivo* experiments (Remy et al., 1977). Stereoselectivity ratios comparable to those found for our compounds were described for a congener of diphenhydramine (123 and 0.4 at M_1 and H_1 receptors) in functional studies (Rekker et al., 1971). Very high inverse stereoselectivity was reported for a set of benzothiazepinone derivatives (3981 and 0.16 at M_1 and H_1, respectively; Eltze et al., 1989). Finally, reports were given concerning inverse stereoselecivity for dimethindene enantiomers (Pfaff et al., 1995) in functional and binding studies at native receptors. This was confirmed in binding studies at $hM_{1\text{-}5}$ and hH_1 receptors stably expressed in CHO-K1 cells (Böhme et al., 2003). Further studies on derivatives of dimethindene were part of this work and will be discussed later.

It can be summarised that inverse stereoselectivity between H_1 and M_1 receptors is not a single phenomenon but may be a more general feature, as it was observed in several structures with a wide chemical diversity. As actually very few subtype-selective muscarinic antagonists are available, racemic H_1 antagonists may serve as a new starting point for SAR research. Resolution of these compounds into pure enantiomers may reveal completely new binding characteristics, which were previously not recognised and may result in new lead structures for the development of selective muscarinic antagonists.

5.5 Analogues of glycopyrronium

The racemic mixture of (RS, SR)-glycopyrronium, named glycopyrrolate, is sold as Robinul®. This drug has a long history as an antimuscarinic agent in pretreatment during general anaesthesia to reduce gastric acid production and salivation, where it is still in use (Ali-Melkkilä et al., 1993; Rautakorpi et al., 1999). First reports showing efficacy of glycopyrrolate as a potent bronchodilator in asthma patients were given in the end of the 1980s (Walker et al., 1987; Schroeckenstein et al., 1988). The first synthetic antimuscarinic drug that was used worldwide as a bronchodilator was the quaternary compound ipratropium (Pakes et al., 1980). This was a step forward in comparison to the formerly used tertiary tropa-alkaloids atropine and scopolamine with regard to the side effect profiles. Due to its quaternary structure, absorption of ipratropium in the GIT and airways is low, resulting in less dry mouth, blurred vision and CNS side effects. In the last ten years, chronic obstructive pulmonary disease (COPD) was recognised as a major problem in public health. As antimuscarinic drug therapy is currently one of the most effective therapies next to symphatomimetic drugs, interest in new compounds increased. Derivatives of glycopyrronium were discovered as highly potent antimuscarinic compounds. SAR studies were already performed in our laboratory by K. Kreutzmann. We continued the examination of recently synthesised derivatives of glycopyrronium at muscarinic receptor subtypes.

5.5.1 Influence of N-alkylation

Several analogues of glycopyrronium with pyrrolidinium structure were tested with modification in N-alkylation. Introduction of an allyl substituent (**43D** → **Dia-44D**) resulted in a approximately 2-fold decrease in affinity at all subtypes. A propargyl substituent with a triple bond (**43D** → **Dia-45D**) showed no change in affinities. One must notice that **Dia-44D** - **Dia-48D** were tested as mixtures of diastereomers with possibly different binding characteristic. Therefore one can speculate that isolation of the pure isomers would provide an eutomer with higher affinity and a distomer with lower affinity. This must be kept in mind when comparing **43D** with these compounds. The eutomer of **Dia-44D** is likely to be equipotent to **43D** and the eutomer of **Dia-45D** may even be more potent. Compound **Dia-46D** with an N-trifluorpropyl substituent had slightly decreased affinities (up to 4-fold at M_2) comparable to the values of the

phenylethyl derivative **Dia-47D** (up to 3-fold). The phenylpropyl congener **Dia-48D** showed comparable affinities as the parent compound **43D**. We further investigated some derivatives with a chinuclidine ring as a basic moiety. The phenylethyl derivative **50D** was equipotent to the methyl substituted congener **49D**. Slightly increased affinity values were found for the phenylpropyl derivative **51D** in comparison to **49D** (2.6-, 5.2-, 1.6-, 1.6- and 1.4-fold at $M_{1\text{-}5}$, respectively).

Taken together, substituents at the basic nitrogen atom were shown to be not critical for binding affinities at $M_{1\text{-}5}$ receptors in derivatives containing a pyrrolidine or chinuclidine ring in our series of compounds. The affinities of all compounds were in the range of that of the parent compound **43D** (values ranging from 9.4 - 10.0, K. Kreutzmann, personal communication). Introduction of aromatic substituents was tolerated without major changes in affinity. Comparable or slightly increased affinities were measured for the phenylpropyl congeners **Dia-48D** and **51D** as an optimum among the tested derivatives in comparison to their N-methyl congeners **43D** and **49D**, respectively.

5.5.2 Influence of the amino-alcohol

A direct comparison of the four stereosisomers of glycopyrronium **43A-D** (Fig. 5.10 (**B**)) with its chinuclinide congeners **49A-D** (Fig. 5.10 (**A**)) was performed. Fig. 5.10 (**C**) gives the differences in affinity values for **43A-D** and **49A-D**. It could be seen that changing the amino-alcohol from a pyrrolidine ring (**43A-D**) into a chinuclidine ring (**49A-D**) led to minor differences with only slightly decreased affinities for compounds **49A-C**. However, for the most important stereoisomer **49D**, affinities were equal or increased. Comparing the pyrrolidine compounds **Dia-47D** and **Dia-48D** with their chinuclidine analogues **50D** and **51D** a slightly increase in affinity was found in favour of compounds **50D** and **51D** displaying a chinuclidine structure. Taken together, slightly improved affinities were measured for chinuclidine analogues in comparison to their pyrrolidine congeners for the (3R, 2'R)-configurated compounds.

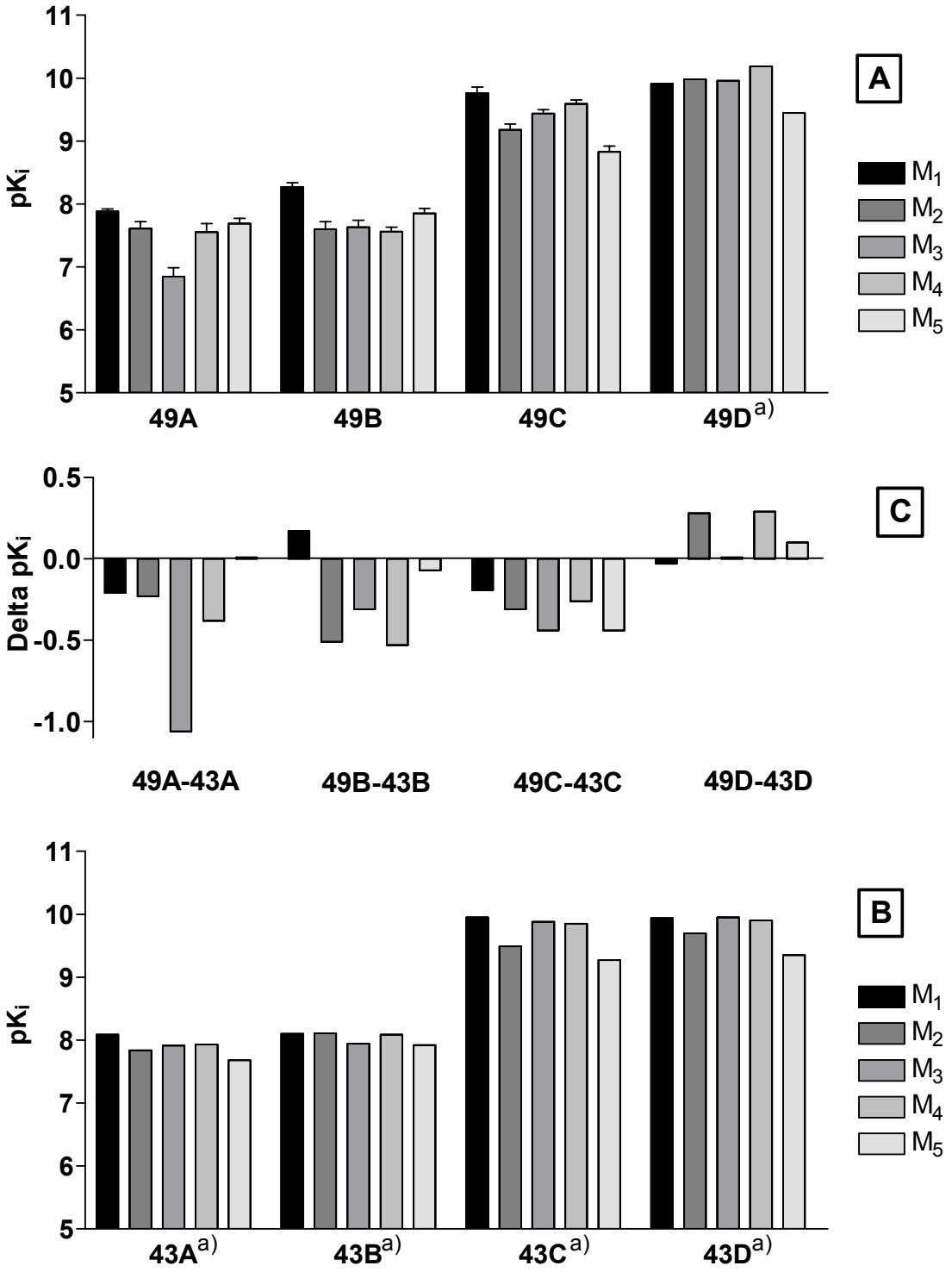


Fig. 5.10 *Affinity values (pK_i) of the chinuclidine derivatives **49A-D** (**A**) and the parent compounds **43A-D** (**B**) and differences between these data sets (**C**) at hM_{1-5} receptors. [a] Values were measured by K. Kreutzmann (personal communication).*

5.5.3 Dimerised molecules and synthesis precursors

Connecting two molecules of **43D** by an alkyl spacer led to compound **52**. Pronounced decreases in affinities were measured (59-fold at M_5 up to 120-fold at M_1). **52** was tested as a diastereomeric mixture. Very interesting were the results obtained with **Dia-53**, lacking the cyclopentyl ring in the acid part. Losses of affinities compared to **43D** were found (between 1047-fold at M_5 and 3802-fold at M_1). As affinities were dramatically decreased, it can be concluded, that an alicyclic ring in 2'-position is a necessary structural feature for high affinity ligands in this series of muscarinic antagonists. Only small differences were observed between **Dia-53** and **(R)-54** (up to 6-fold), showing the exchange of a hydroxy group into a ketone function being of little influence.

5.5.4 Tiotropium - glycopyrronium hybrids

Tiotropium has been reported to be a highly potent antagonist at muscarinic receptor subtypes in binding studies in several human tissues (Haddad et al., 1994). Unfortunately, never a full paper was published concerning the binding affinities of tiotropium at recombinant human receptors. It was of interest to investigate hybrid molecules consisting in parts of chemical properties of glycopyrronium and tiotropium to obtain new information about the pharmacophore at muscarinic receptor subtypes. Compound **(R)-55** consisted of the acid part of glycoyrronium and the tropanyl substituent of tiotropium. This compound had marginally decreased affinities in comparison to the pyrrolidinium parent compound **43D** at the most subtypes (up to 4-fold) and a 2-fold increase at M_2. Building up an affinity rank order for different N-methyl-substituted compounds containing the acid part of the parent compound **43D** we found: **(R)-55** < **43D** < **49D** = tropanyl < pyrrolidine < chinuclidine. However, the differences of affinities were only small. Interestingly, compound **(R)-56** with the achiral dithienyl acid part of tiotropium had equal affinities to its congener with the glycopyrronium acid part, **50D**. We conclude that the acid part of tiotropium and glycopyrronium are exchangeable concerning binding affinity at $M_{1\text{-}5}$ receptors and that it is of minor importance, whether there is an alicyclic or an aromatic substituent attached in 2'-position in the acid part of the molecules.

5.5.5 Stereochemical aspects

Highly interesting was to notice the influence of the absolute configuration at the two chiral centres of compounds **43A-D** and **49A-D**, for which all 4 possible stereoisomers were tested (**43A-D**, and **49D** by K. Kreutzmann, personal communication). We found for both sets of derivatives an at least 2 orders of magnitude higher affinity for isomers with a (2'R)-configuration (compounds **C** and **D**) in comparison to the (2'S)-configurated analogues (compounds **A** and **B**). In addition, only minor influence of the absolute configuration in the amino-alcohol on affinity was observed in both series. Compounds **C**, **D** and, respectively **A**, **B** had almost identical affinities at $M_{1\text{-}5}$ receptors. We conclude that the chiral centre in pyrrolidinium and chinuclidnium amino-alcohols, tested in our series, was not a very critical part concerning equilibrium binding affinity. Similar findings concerning the preference of (2'R)-configurated benzil acid derivatives at muscarinic receptor subtypes has been reported. In a series of ether analogues with tropanyl amino-alcohol structures, the (2'R)-configurated compounds were at least one order of magnitude more potent than their (2'S)-congeners (Gao and Liu, 1995). In this series, influence of the second centre of chirality located in the basic structure was of minor importance, as seen in our series. Reports on chinuclidine-ester of benzil acid analogues showed the (2'R)-configurated enantiomers to be more potent, too (Kiesewetter et al., 1995). Interestingly, the (3R, 2'R)-isomer was shown to possess a selectivity for the M_1 subtype. We could not confirm this finding in our series.

Taken together, our data confirm the importance of absolute configuration at the chiral centre in the acid part of the molecules for affinity to muscarinic receptor subtypes. The (2'R)-configurated compounds were generally reported to be more potent compared to there (2'S)-stereoisomers. Only little influence was seen for the absolute configuration in the basic amino moiety. In our series the following ranking was found: **D** $\geq$ **C** >> **B** = **A**.

5.6 Characterisation of [^{3}H](3R, 2'R)-glycopyrronium

Diagnosis of COPD is becoming more and more frequent at this time and is expected to become one of the most important therapeutic problems in the near future. However, only symptomatic therapy is currently possible, improving quality of daily life and reducing exacerbations, wherein antimuscarinic agents build the backbone in therapy (Campillo and Paez, 2002). Only few antimuscarinic drugs are available at the moment for inhalation therapy. All these medicinces are quaternary compounds which are poorly absorbed in human airways and the GIT, resulting in very few systemic side effects next to dry mouth (Corne and Anthonisen, 2002; Beeh et al., 2002). In preliminary studies with unlabelled, cold drugs carried out in our laboratory, **43D**, the (3R, 2'R)- stereoisomer of glycopyrronium turned out to be one of the most interesting compounds, being a highly potent muscarinic antagonist displaying kinetic selectivity for M_3 receptors. In the following paragraphs, binding profile and kinetic properties of the labelled compound [^{3}H]43D will be discussed next to other compounds currently in use in the field of COPD therapy with a special focus on ipratropium and tiotropium as reference drugs. Additionally, a comparison with previous data obtained in our laboratories in functional and binding experiments is given.

5.6.1 Muscarinic receptors in human airways

In human airways, $M_{1\text{-}3}$ receptors were identified and their function was extensively investigated (Barnes, 1993; Caulfield and Birdsall, 1998; Eglen et al., 2001; Corne and Anthonisen, 2002).

Fig. 5.11 shows the cholinergic innervation found in human airways. M_3 receptors were detected on smooth muscles being most important for direct parasympathetic bronchoconstriction (Bymaster et al., 2003). Additionally, M_3 receptors mediate mucus secretion in human airways. M_2 receptors were identified on cholinergic neurons mediating a negative feedback loop on endogenous ACh release and on smooth muscle cells reversing the sympathetic β-adrenergic tone. M_1 receptors were detected in the soma-dendritic region of postganlionic cholinergic neurons facilitating excitation. With knowledge of the function of each subtype it is of greatest interest for COPD management to achieve a prolonged blockade of M_3 receptors without

blocking the prejunctional M_2 autoreceptors, because this would result in increased ACh release possibly counteracting the postjunctional M_3 blockade.

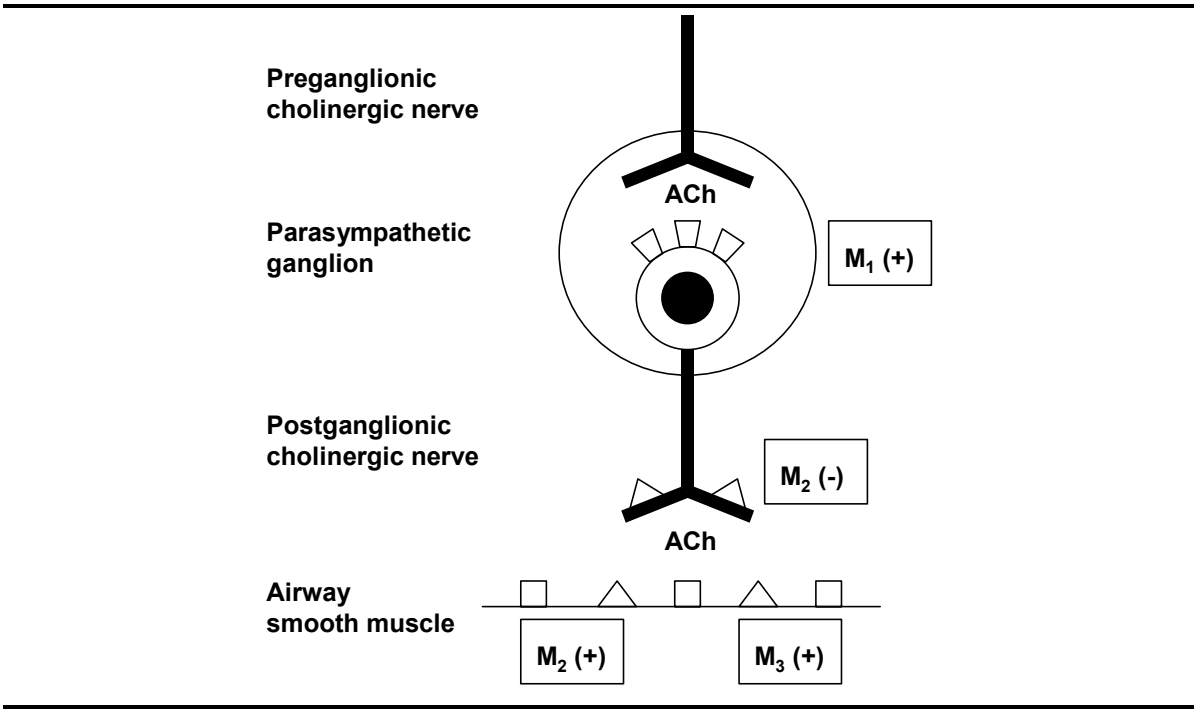


Fig. 5.11 *Cholinergic innervation in human airways (adapted from Hansel and Barnes, 2002). (+) = neuronal excitation or increase of smooth muscle tone; (-) = inhibitory influence.*

M_2 receptors controlling the endogenous ACh release were found to be fully functional in stable COPD (On et al., 2001). However, reports showed the M_2 receptors to be dysfunctional in viral infections causing acute exacerbations in asthma and COPD patients (Jacoby and Fryer, 1999; Fryer et al., 1999). Receptor deglycosylation by viral neuraminidase and release of major basic protein from eosinophils were demonstrated to change M_2 function. M_3 receptors were found to be unchanged in patients with hyperresponsiveness in airway smooth muscles (Fryer and Jacoby, 1998). These findings may account for potentiated reflex bronchoconstriction in patients. Reports concerning paradoxical bronchoconstriction following application of ipratropium were published (Eglen and Watson, 1996; Corne and Anthonisen, 2002). This may be due to blockade of prejunctional M_2 receptors leading to increased ACh release, reducing effectiveness of postjunctional M_3 antagonism. Interesting findings were reported about the nondepolarising neuromuscular blocking agent rapacuronium, used to block nicotinic ACh receptors

to facilitate intubation during anaesthesia. This drug was removed from the market due to high incidence of bronchospasm resulting in deaths. Recently it was demonstrated that the reason for this bronchoconstriction was a selective blockade of M_2 autoreceptors in the airways (Jooste et al., 2003).

5.6.2 Binding profile at $M_{1\text{-}5}$ receptors

As shown (Fig. 4.25 and Fig. 4.26) saturation binding isotherms of [^{3}H]43D were in good agreement with a one-site binding model according to the law of mass action. The obtained K_D values (Table 4.10) were in the range of the affinity values (pK_i) found with the cold drug previously in our lab in competition binding experiments with [^{3}H]NMS (K. Kreutzmann, personal communication). The labelled drug's K_D values (9.67 - 10.55) were approximately by factor 3 higher than the cold drugs pK_i values (9.35 - 9.95) (Fig. 5.12). No pronounced selectivity was seen in binding affinities to $M_{1\text{-}5}$ receptors.

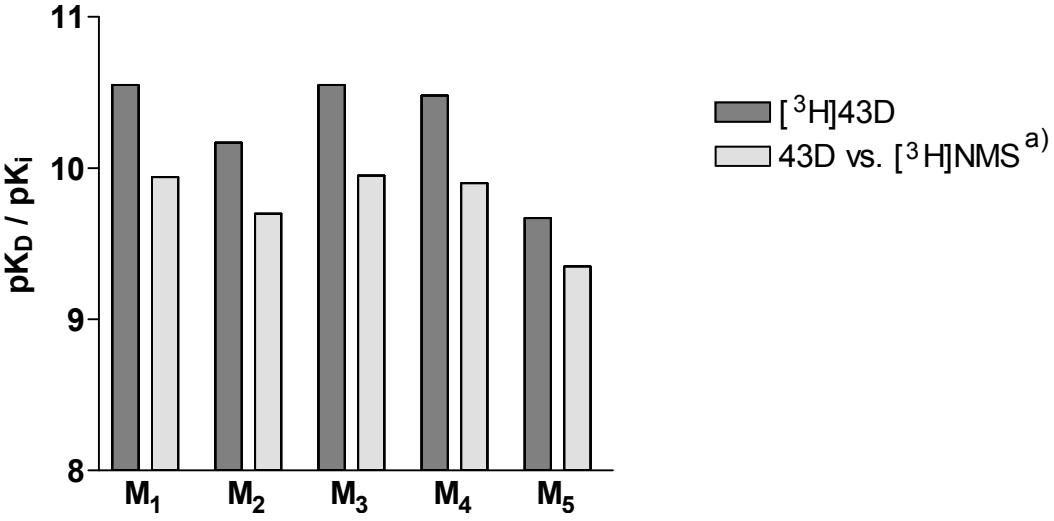


Fig. 5.12 *Affinity values (pK_D) of labelled [^{3}H]43D determined in saturation binding experiments and affinity values (pK_i) of cold **43D** determined in competition experiments with [^{3}H]NMS at $hM_{1\text{-}5}$ receptors stably expressed in CHO-K1 cells.* [a)] *Data from K. Kreutzmann (personal communication).*

In comparison to affinity values of ipratropium recorded in competition experiments with [^{3}H]NMS, an at least 9-fold higher affinity was measured for [^{3}H]43D at

muscarinic receptor subtypes. Our values for ipratropium (Fig. 5.13) were in good agreement with previously reported data (Haddad et al., 1999).

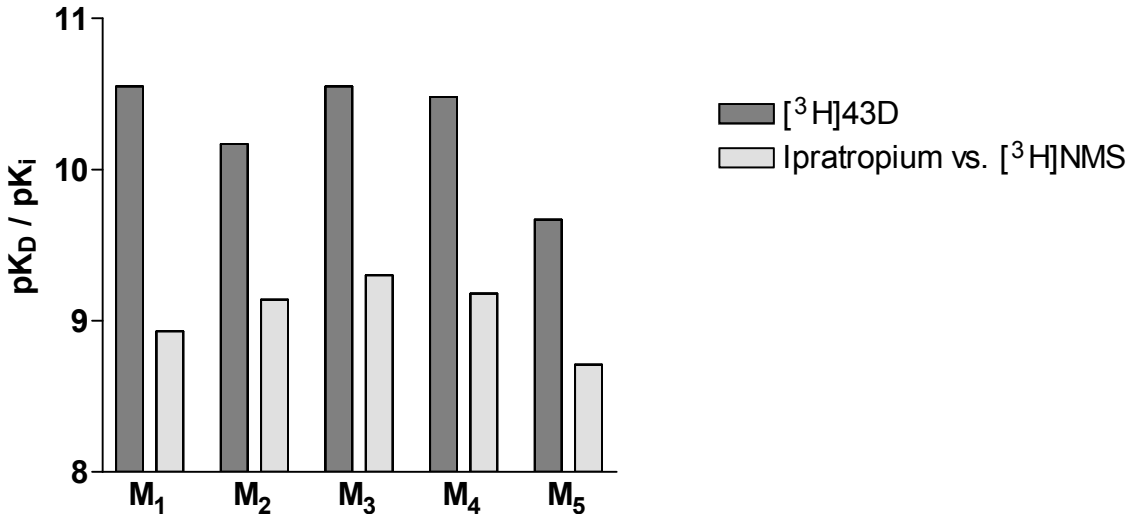


Fig. 5.13 *Affinity values (pK_D) of labelled [^{3}H]43D determined in saturation binding experiments and affinity values (pK_i) of cold ipratropium calculated in competition experiments with [^{3}H]NMS at hM_{1-5} receptors stably expressed in CHO-K1 cells.*

No complete comparison of the affinity profiles at muscarinic receptor subtypes of tiotropium and **43D** could be done, since no full paper reports a complete affinity profile of tiotropium at M_{1-5} receptors. However, for [^{3}H]tiotropium, pK_D values of 9.57, 9.92 and 9.48 at CHO cells expressing M_{1-3} receptors were reported (Disse et al., 1993). This showed tiotropium to be less potent than **43D** at these subtypes.

To check the usefulness of [^{3}H]43D as a novel muscarinic radioligand, we performed a comparative study of binding characteristics of several reference drugs with [^{3}H]43D and [^{3}H]NMS. In Fig. 5.14 (**A**) affinities for the non-selective compound atropine, the M_1-selective drug pirenzepine, the M_2-preferring drug (S)-dimethindene and the $M_{2/4}$-selective compound himbacine are given in studies with [^{3}H]43D. In Fig. 5.14 (**B**) values in studies with [^{3}H]NMS are given. As shown in Fig. 5.14 (**C**), almost no difference was found between the measured affinities at M_{1-5} receptors with the two radioligands. It is concluded that [^{3}H]43D recognised the same binding site as other muscarinic ligands and may serve as a useful radioligand in future studies.

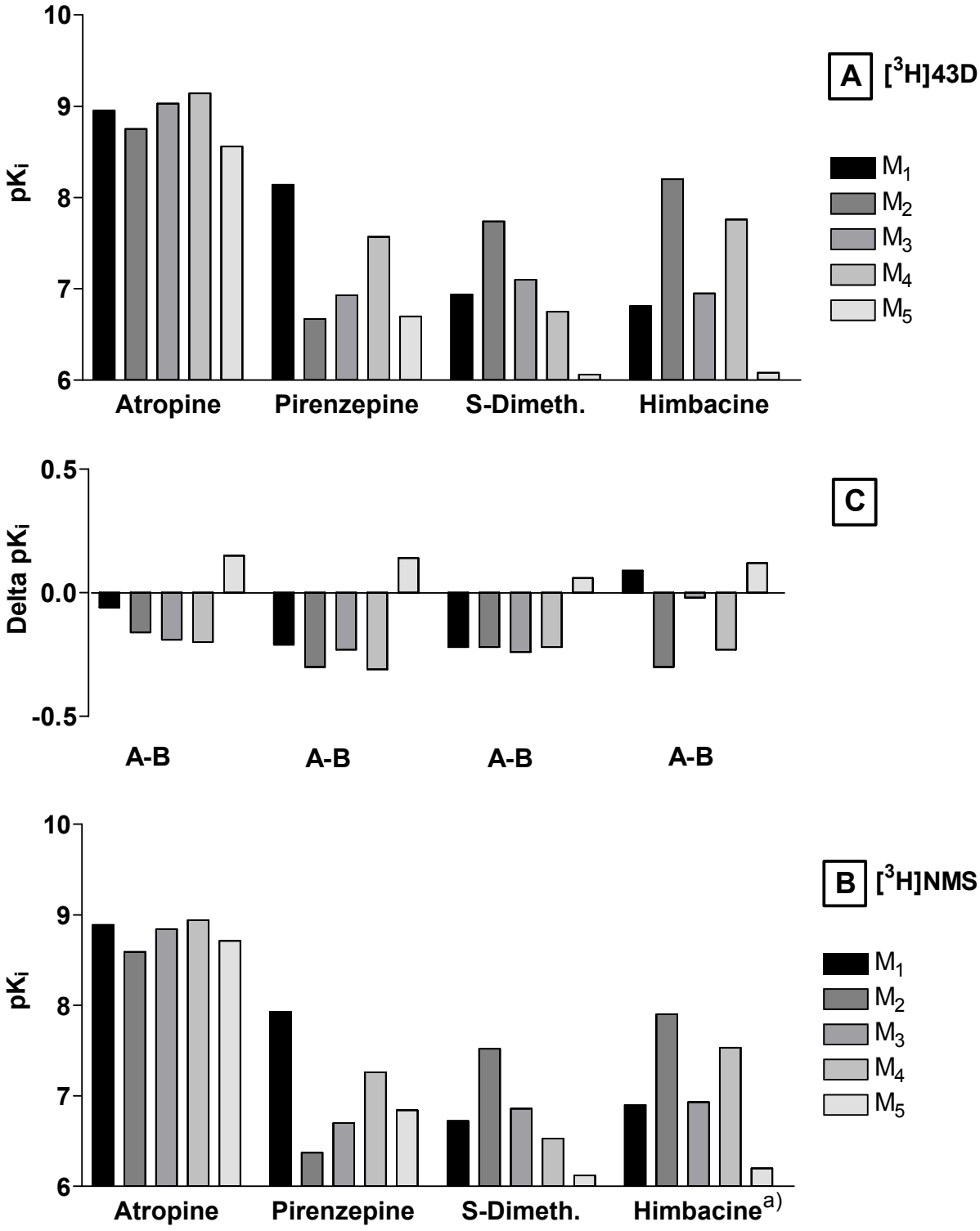


Fig. 5.14 *Affinity values (pK_i) of reference drugs recorded in competition binding experiments with [^{3}H]43D (**A**) or [^{3}H]NMS (**B**) and the differences between the calculated pK_i values (**C**). [a)] Data measured by Dr. C. Keim (personal communication).*

5.6.3 Kinetic properties

In our kinetic binding studies with [^{3}H]43D we were able to confirm the findings of previous research in our laboratory with the unlabeled drug **43D**. A dissociation half-life of 17 and 131 min was determined by K. Kreutzmann at M_2 and M_3 receptors (personal communication). We found almost identical $t_{1/2}$ values of 23 and 145 min at these subtypes with the labelled compound [^{3}H]43D. Dissociation at M_3 was 6-fold slower in comparison to the M_2 subtype resulting in a kinetic selectivity for the M_3 receptor. The following rank order concerning dissociation half-lifes was found: M_3 > M_5 > M_4 > M_1 >> M_2. For [^{3}H]ipratropium, and [^{3}H]tiotropium dissociation half-lifes were reported at M_{1-3} receptors stably expressed in CHO cells (Disse et al., 1993). For ipratropium a rapid dissociation was found with 6.6, 2.1 and 16 min at M_{1-3} receptors, respectively. Much slower dissociation was determined for tiotropium with 15, 3.6 and 35 hours (!) at M_{1-3}, respectively. This equals a 8- and 10-fold slower dissociation at M_3 versus M_2 for ipratropium and tiotropium. Findings for tiotropium are in contrast to the results published in studies at human lung tissue preparations, labelling M_1 and M_3 receptors (Haddad et al., 1994). A simple exponential function provided the best description for the measured data and no biphasic dissociation profile was seen. The calculated half-life was 414 min. This value is neither in agreement with the previously published value for M_1 (15 h) nor with that for the M_3 subtype (35 h). No explanation for this discrepancy is given by the authors. In preliminary experiments in our laboratory with tiotropium at M_3 receptors, a dissociation half-life of 212 min was found which is in range of the values found in human lung tissue (K. Kreutzmann, personal communication). Reasons for these discrepancies remain unclear as kinetic data for tiotropium were never published in a full paper. For ipratropium, **43D** and tiotropium a slower dissociation was found at M_3 versus M_2. However, for ipratropium dissociation at M_3 was fast, too. Therefore, no kinetic selectivity for M_3 receptors resulted for this compound. **43D** had a much slower dissociation than ipratropium at M_3 (145 versus 16 min) resulting in prolonged time of action at this subtype. Compared to tiotropium, dissociation was more rapid. Due to the discrepancies between the reported values for tiotropium it remains to be elucidated how great the difference really is in fact between the dissociation half-life at M_3 for **43D** and tiotropium.

Using the results of our kinetic experiments we calculated the kinetic K_D values ($kinK_D$) for [^{3}H]43D as described in material and methods. $kinK_D$ values were almost identical to those measured in saturation binding experiments ($satK_D$). Fig. 5.15 gives a comparison of $kinK_D$, $satK_D$ obtained with [^{3}H]43D and pK_i values derived in competition experiments with unlabelled **43D** in competition binding studies with [^{3}H]43D and [^{3}H]NMS as radioligands.

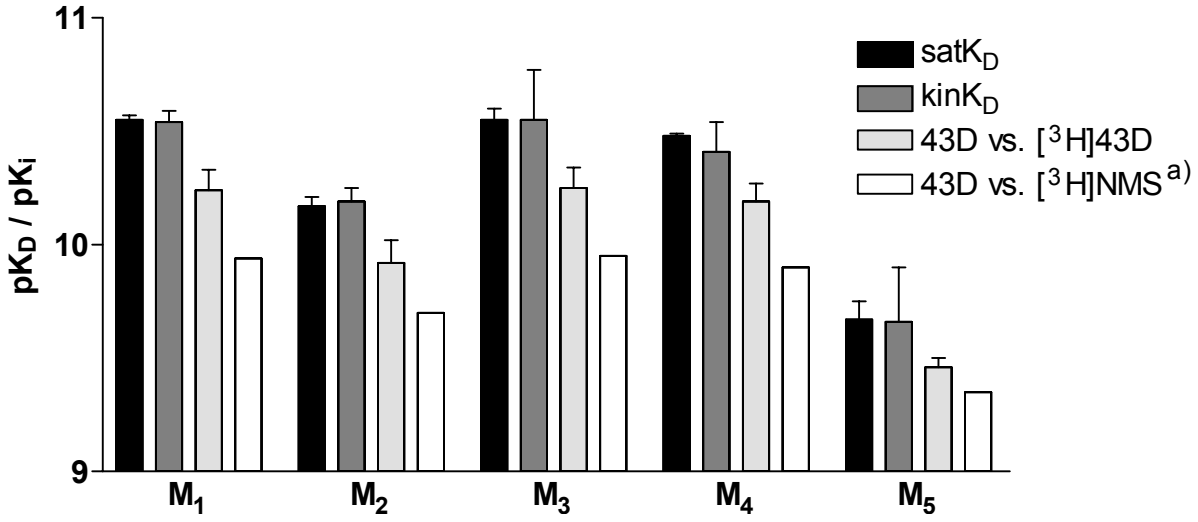


Fig. 5.15 *Comparison of $kinK_D$, $satK_D$ obtained with [^{3}H]43D and pK_i values derived in competition experiments with cold **43D** in competition binding studies with [^{3}H]43D and [^{3}H]NMS. [a] K. Kreutzmann (personal communication).*

K_D values determined in saturation studies were absolutely identical to those calculated in kinetic studies, proving the high validity of the kinetic data. pK_i values calculated for **43D** in competition experiments with [^{3}H]43D were approximately 2-fold below the obtained K_D values. Competition data with [^{3}H]NMS were approximately 3-fold below K_D values. Taken together, binding data obtained in competition and kinetic experiments are in good agreement with each other and prove a good validity of our data.

5.6.4 SAR based on kinetic properties

Due to experimental complexity and demand on time only little is known about SAR concerning kinetic properties of compounds at a special receptor site. To obtain kinetic binding constants of a compound there are two possible ways: Firstly, using a

radioactively labelled molecule which is extremely expensive. Secondly, performing highly sophisticated cold kinetic experiments and fitting of the obtained data to complex mathematical equations, describing the influence of an unlabelled drug on the known binding kinetic properties of a radioligand. However, both methods demand great expense of time and material. In our laboratory research at muscarinic receptors with unlabelled drugs was carried out by K. Kreutzmann with the stereoisomers of **43** and several derivatives. He found the following dissociation half-lifes for **43A-D** at M_3 receptors: < 1, 2, 75 and 131 min, respectively. The (2'R)-configurated stereoisomers **43C** and **43D** dissociated much slower than their (2'S)-congeners. Based on these findings patents were filed worldwide with the (3R, 2'R)-isomer of **43** and several analogues, as compounds with kinetic selectivity at M_3 receptors. In the following time, research with several (3R, 2'R)-configurated analogues of **43** possessing piperidine and pyrrolidine ring systems as basic moiety was done. However, no prolonged time of action was achieved. Recently, a series of chinuclidinol esters was claimed in a patent from Almirall Prodesfarma as long acting, kinetic selective M_3 antagonists for use in COPD ([Anon], 2003). In our laboratory, the chinuclidinol analogue **49D** was examined at M_3 receptors and an extremely slow dissociation rate at M_3 receptors was found (364 min, K. Kreutzmann, personal communication). This compound had the slowest dissociation half-life of all compounds tested in our laboratory and is the most interesting candidate for further development as a potential drug in COPD therapy. Dissociation half-life of **49D** was 2.5-fold increased in comparison to **43D** which was completely characterised in this work. The influence of stereochemistry on kinetic properties was impressively demonstrated in our series of compounds at muscarinic receptors. Recently, an interesting finding was reported for the well known racemic H_1 antagonist cetirizine. Separation into enantiomers resulted in two completely different acting compounds concerning the dissociation half-life at H_1 receptors. (R)-cetirizine was reported with a $t_{1/2}$ of 142 min, whereas (S)-cetirizine had only a $t_{1/2}$ of 6 min (Gillard et al., 2002).

Taken together, in our series of compounds we demonstrated a dramatic influence of stereochemistry on kinetic properties at muscarinic receptor subtypes. Size and shape of the basic moiety are important as well as the substituents in the acid part of the molecules. As some modifications are tolerated (compare tiotropium versus **43D** and **49D**) further studies are necessary to clearly demonstrate the influence of

special parts of the tested molecules on binding kinetics at a special receptor subtype.

5.6.5 Functional studies

In previous work in our laboratory, the stereoisomers of **43** were investigated in functional studies in rabbit vas deferens (M_1), guinea-pig atria (M_2) and guinea-pig ileum (M_3). In contrast to atropine, ipratropium and **43A**, compounds **43C**, **43D** and glycopyrrolate (which is a racemic mixture of **43B** and **43C**) did not behave as simple competitive muscarinic antagonists at muscarinic M_3 receptors (Czeche, 2000). A concentration-dependent decrease in maximum response to the agonist arecaidine propargyl ester was observed and a very slow offset of action, determined as time to recover 100% response to agonist after washout of the antagonist. The determined kinetic data may serve as a reasonable explanation for these findings. For the (3R, 2'R)-configurated compound **43D** a very slow dissociation was shown in cold kinetic experiments (131 min, K. Kreutzmann, personal communication) which was confirmed in our studies with [^{3}H]43D (145 min). Additionally, in cold kinetic experiments an extremely fast dissociation was found for the (2'S)-configurated compounds **43A** and **43B** ($t_{1/2}$ < 1 and 2 min, respectively) and an intermediate value for **43C** ($t_{1/2}$ = 75 min) was found (K. Kreutzmann, personal communication). It seems, as if the slow dissociation profiles of the (2'R)-configurated analogues **43C**, **43D** account for the non-selective behaviour at M_3 in functional studies. In the case of glycopyrrolate the isomer **43C** (present in this racemic mixture of **43B** and **43C**) is likely to account for these findings. Functional data for **43B** are not further discussed as an impurity with **43D** was found later on. Previously reported functional data with glycopyrrolate were not in good agreement with those obtained in our laboratories (Lau and Szilagyi, 1992; Fuder and Meincke, 1993) which is possibly due to impurities with the other stereoisomers (**43A** and **43D**). Results of functional studies carried out in our laboratory were in good agreement with our findings in binding experiments, confirming a kinetic selectivity at M_3 receptors. An interesting study was carried out comparing glycopyrrolate with ipratropium in functional studies in guinea-pig and human airways (Haddad et al., 1999). Glyopyrrolate was more potent and had a longer duration of action than ipratropium, most likely due to the slow dissociating isomer **43C**.

5.6.6 *In vivo* studies

Only little data is available concerning **43D** in animal studies. Some studies were carried out at the Department of Pharmacology 2, Arzneimittelwerk Dresden GmbH, Radebeul, Germany. Results were reported by Prof. Dr. I. Szelenyi in personal communication to Prof. Dr. G. Lambrecht. The potency and duration of action of **43D** were determined in a methacholine-induced bronchoconstriction model in anaesthetised guinea-pigs. An intravenous bolus application of methacholine (20 µg/kg) was given and the change of bronchial response before and after application of **43D** was determined as percent change of lung resistance. In a first study methacholine was repeatedly applicated intravenously in 30 min intervals for 4 h. In a second study arm, guinea-pigs were pretreated with **43D** i.m. 18 h before methacholine challenge. Glycopyrrolate served as a reference drug. It was shown that **43D** at a dose of 0.3 µg/kg i.v. potently inhibited the increase in lung resistance induced by methacholine. This effect was more pronounced compared to glycopyrrolate at a dose of 1.0 µg/kg intravenous. Very interestingly, 18 h after pretreatment with **43D** at 1.0 µg/kg i. m., there was still a 58% inhibition of the methacholine effect, whereas glycopyrrolate at 3.0 µg/kg i.m. showed a much lower effect (36%). In addition, in the same model of bronchoconstriction a shorter time of action was measured for ipratropium in comparison to **43D** (Pfister et al., 1985). Taken together, in vivo studies showed **43D** to be a highly potent muscarinic antagonist with a prolonged time of action compared to ipratropium and glycopyrrolate.

Much more data is available for glycopyrrolate which is in clinical use to reduce salivation and release of gastric acid in general anaesthesia (Ali-Melkkilä et al., 1993; Rautakorpi et al., 1999). Studies concerning pharmacokinetic and pharamcodynamic properties were recently reported (Penttilä et al., 2001). Research concerning its use as a bronchodilator was carried out. A prolonged effect up to 12 h was seen in asthma patients (Walker et al., 1987; Schroeckenstein et al., 1988). In COPD, benefit to patients of glycopyrrolate was shown, too (Tzelepis et al., 1996).

5.6.7 Therapeutic implications

Since the introduction of ipratropium in the late 1970s only very few compounds were added to the market for inhalation therapy as antimuscarinic drugs. At present, two non-selective compounds, ipratropium and oxitropium, are in use, needing a 4-times and 3-times daily dosing regimen of 200 µg in general. With the introduction of tiotropium a step forward was made in therapy. A once daily dosing regimen with 18 µg was sufficient to obtain superior bronchodilation in comparison to the older drugs in use (Disse et al., 1993; Barnes et al., 1995; Panning and De Bisschop, 2003). Due to better efficacy and a more convenient dosing regimen it is likely that tiotropium will become more important than the older drugs. Tiotropium was shown to be a non-selective compound as well as the older drugs in equilibrium binding experiments. However, due to its slow dissociation at the M_3 subtype a kinetic selectivity and prolonged time of action was achieved. Studies with glycopyrrolate showed already superiority to ipratropium in functional experiments in guinea-pig and human airways as a bronchodilator (Haddad et al., 1999) with a prolonged time of action. One should notice, that the enantiomer **43C** (which is most likely accounting for the prolonged time of action in this studies) had only a dissociation half-life of 75 min at M_3 receptors. As written in 5.6.6 **43D** was superior in animal studies to glycopyrrolate. Therefore it is likely that studies with **43D** (dissociation half-life 145 min at M_3) will result in an additional gain in the duration of action. It will be very interesting to see the results of the first clinical trials in humans carried out with **43D**. One can expect a duration of action of at least 12 h, allowing a twice daily dosing, perhaps a once daily dosing comparable to tiotropium.

Further therapeutic fields apart from COPD were investigated with glycopyrrolate. Studies on topical use for gustatory sweating in diabetic patients (Frey syndrome) were carried out (Hays et al., 1982; Atkin and Brown, 1996; Shaw et al., 1997) showing good results. Perhaps further studies with **43D** will bring up even better results not only for use in diabetic patients but in hyperhidrosis in general. This would be an interesting new therapeutic target as topical appliation of $AlCl_3$ preparations show little efficacy and application of botulinum toxin is very expensive. Additionally, a potential use in bladder dysfunction and urinary incontinence seems possible. Sepracor Inc. has filed use patents for the four isomers of **43** for bladder dysfunction (Butera and Argentieri, 1998).

5.7 M_2-selective antagonists related to dimethindene

5.7.1 Modification of side chain length

As mentioned above, the most difficult task in the development of M_2-selective antagonists related to dimethindene was to increase the affinity to the M_2 subtype and maintain high selectivity and specificity. In a series of analogues with reduced side chain length (n=1) we found an increase in affinity to M_2 receptors for all tested compounds in comparison to their analogues with normal chain length (n=2). Fig. 5.16 gives a comparison of affinity values at M_2 and H_1 receptors in a series of analogues tested with n=2 ("**A**" series) and n=1 ("**B**" series) (see Table 2.23). In addition to the increased affinity to M_2 receptors, affinity to H_1 receptors was reduced with exception of **59B** where a slightly increased H_1 affinity was determined. Increase in affinity to the M_2 subtype lay between approximately 3 - 10-fold, whereas variability was observed at the decrease in affinity to the H_1 subtype.

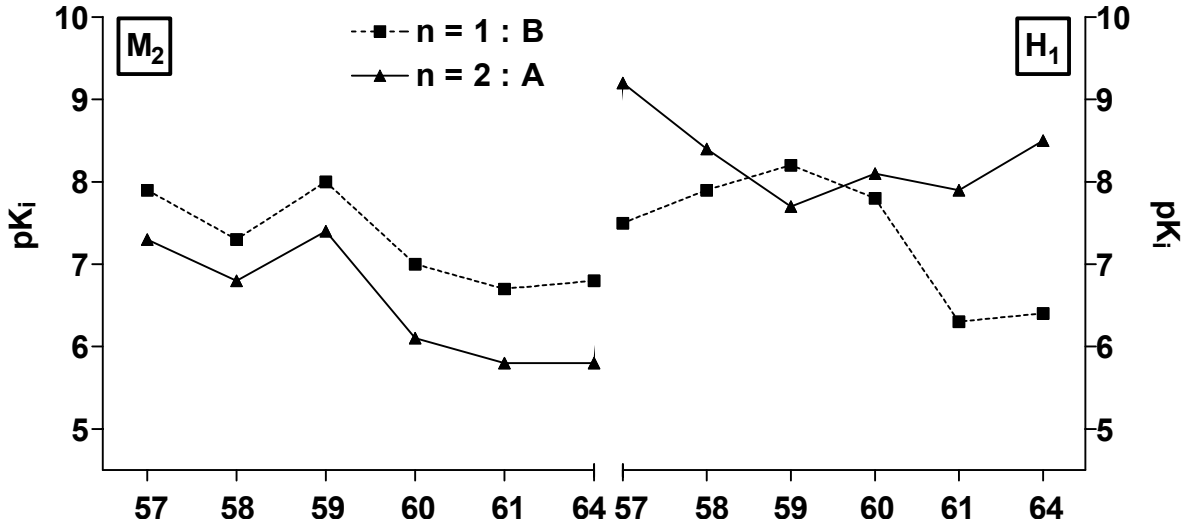


Fig. 5.16 *Affinity values (pK_i) to M_2 (left) and H_1 (right) receptors for a set of compounds tested with n = 2 ("**A**" series, solid line) and n = 1 ("**B**" series, dashed line) (see Table 2.23).*

In summary, reduction of side chain length to n=1 resulted in increased affinity to muscarinic receptor subtypes and at the same time decreased H_1 affinity in most cases. Unfortunately, the n=2 congener of the most interesting compound within this series **72B**, was not available until the end of this work.

5.7.2 Modifications at the amino moiety

As a consequence of the above findings, several compounds were synthesised with reduced chain length and various modifications of the substitution pattern at the basic amino moiety. Compound **72B** with an isopropyl and a phenylethyl substituent in the basic moiety was found to be an optimum within our series of compounds, with high affinity to M_2 receptors, good selectivity and excellent specificity versus the H_1 subtype. All tested modifications led to reduced affinity or decreased selectivity. This was also true for the closely related compounds **76B**, with a fluoroethyl instead of the isopropyl substituent, and **77B**, with an additional p-methyl group in the phenylethyl ring. Thus, it is concluded, that size and shape of substituents attached to the amino moiety are highly critical to binding characteristics. It is also noteworthy, that tested compounds of the "B" series with an aromatic substituent at the basic nitrogen had comparably low affinities to the H_1 subtype (all below pK_i = 6.46). Additionally, introduction of halogen substituents in m- or p-position of the phenylethyl moiety (**78B** - **83B**) led to decreased affinity to M_2 and reduced selectivity in comparison to **72B**, too.

5.7.3 Stereochemical aspects

Three compounds were examined as pure enantiomers. These were the parent compound **57A**, the diisopropyl congener **75A** and the most interesting compound **72B**. Affinity to the M_2 subtype, selectivity versus $M_{1,3,4,5}$ and specificity versus H_1 for the eutomers at muscarinic receptor subtypesare shown in Table 5.2.

Table 5.2 *Affinities to M_2 receptors of the eutomers at muscarinic subtypes* ***(+)-57A****,* ***(-)-75A*** *(Böhme et al., 2003) and* ***(+)-72B*** *and calculated subtype selectivity and specificity ratios.*

No.	pK_i M_2	M_2 / M_1	M_2 / M_3	M_2 / M_4	M_2 / M_5	M_2 / H_1
(+)-57A	7.52	6	5	10	25	2
(-)-75A	7.37	36	95	42	275	38
(+)-72B	8.59	79	102	31	120	1349

As can be seen in Table 5.2, compound **(+)-57A** had little selectivity and no specificity versus H_1. The diisopropyl congener **(-)-75A** revealed much better selectivity and specificity. However, the absolute affinity to M_2 receptors was not increased. In **(+)-72B** good selectivity was maintained and at the same time a 17-fold increase at affinity to M_2 receptors was achieved with superior specificity (1349-fold versus H_1). With **(+)-72B** a highly potent and selective M_2 antagonist was found.

For the enantiomers of these compounds stereoselectivity were calculated (Table 5.3). These values ranged from 3 - 41 for muscarinic receptors and 0.006 at H_1 for the parent compound **57A**. Comparable results were found for **75A** and **72B** with highest stereoselectivity ratios within the muscarinic family calculated for the M_2 subtype and inverse stereoselectivity at H_1 receptors.

Table 5.3 *Stereoselectivity ratios for enantiomers of **57A**, **75A** and **72B**.*

No.	M_1	M_2	M_3	M_4	M_5	H_1
57A	10	41	25	13	3	0.006
75A	3	18	2	6	6	0.2
72B	4	13	5	3	0.4	0.07

Fig. 5.17 summarizes the M_2 and H_1 affinities of **57A**, **75A** and **72B** and their enantiomers. The eutomer at muscarinic receptors is placed to the left of the corresponding racemic mixture, the eutomer at H_1 receptors on the right hand side. In theory, a maximum 2-fold increase in affinity can be observed from the racemic mixture to the eutomer. That was exactly found for the parent compound at M_2 and H_1 receptors (Fig. 5.17 left side). In the case of **75A** (Fig. 5.17 middle) the racemic mixture was more potent than the pure enantiomers. The muscarinic eutomer was slightly less potent than the racemate, the eutomer at histamine receptors was equipotent. This might be due to chemical instability of the free bases of the pure enantiomers tested. For **72B** (Fig. 5.17 right hand side) the muscarinic eutomer was slightly more potent than the racemic mixture. For the eutomer at H_1 receptors a 6-fold increase was calculated with regard to the racemate.

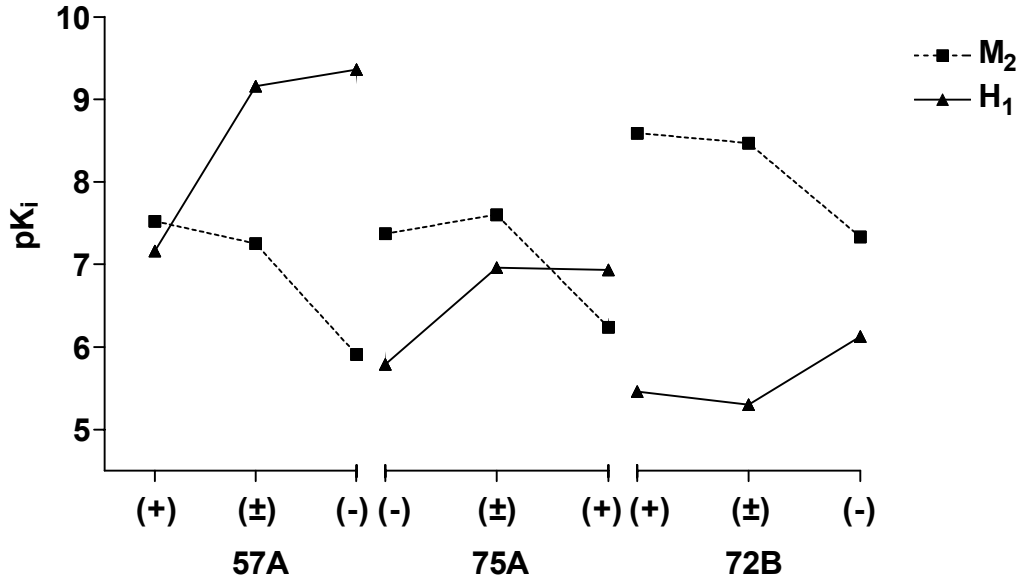


Fig. 5.17 *Affinity values (pK_i) of racemic compounds **(±)-57A**, **(±)-75A** and **(±)-72B** and the pure enantiomers at muscarinic M_2 receptors (dashed line) and histamine H_1 receptors (solid line).*

5.7.4 Comparison of 72B with other M_2-selective antagonists

With the discovery of the M_4 subtype several formerly considered M_2-selective compounds were shown to be unable to discriminate between M_2 and M_4 receptors, for example himbacine, AF-DX384 and methoctramine (Dörje et al., 1991). **72B** is one of to the most potent M_2-selective compounds published to date with good selectivity, only exceeded by some compounds recently synthesised by Schering-Plough (Wang et al., 2002a). Fig. 5.18 gives a comparison to some of the strongest competitors in the field of M_2-selective compounds.

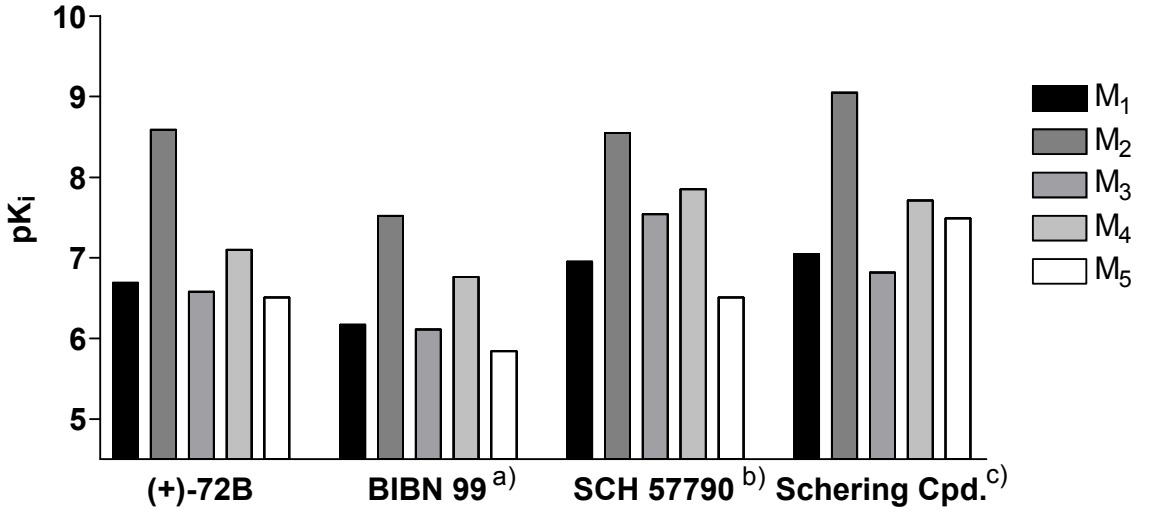


Fig. 5.18 *Affinity profiles of selected M_2-selective antagonists determined in radioligand binding studies at $M_{1\text{-}5}$ receptors. [a)] Doods et al., 1993; [b)] Lachowicz et al., 1999; [c)] Wang et al., 2002a.*

In summary, **(+)-72B** can be considered as a major break-through in our studies of development of M_2-selective antagonists related to dimethindene. At present, **(+)-72B** is together with some recently published compounds by Schering-Plough one of the most potent, highly selective M_2 antagonists worldwide.

5.7.5 Therapeutic implications

With **(+)-72B** not only an interesting pharmacological tool was discovered but also a potential drug for use in AD. M_2-selective antagonists were shown to facilitate ACh release in the brain of several animals. Furthermore, improvement of cognition was demonstrated in a couple of animals from rat to monkey (Lachowicz et al., 2001; Carey et al., 2001). However, a therapeutic long-term benefit in human AD therapy is questionable as cholinergic neurons die with proceeding of AD (Sheardown, 2002). Further studies in humans are needed to verify, whether M_2 antagonists provide a useful therapeutic approach in AD or not. Tachycardia as a side effect might be a problem. Animal studies with SCH57790 showed an increase in cognition at drug doses well beneath cardiac effects. Perhaps a combination therapy with M_2-selective antagonists and AChE inhibitors will result in a synergistic benefit in AD. Apart from therapeutic implications per se, **(+)-72B** might be an interesting tracer for PET

studies, quantifying M_2 receptors in the brain. Unfortunately, all tested derivatives with a fluorine atom in the molecule (to be exchanged into a ^{18}F in a PET tracer) were less potent and/or selective. Studies carried out by radiochemists may find a way to label the isopropyl group (with ^{11}C) of **(+)-72B**. An M_2-selective PET-tracer would be a milestone in AD as a safe *in vivo* diagnosis might become possible. Additionally, monitoring of success or failure of new disease modifying therapies to slow down AD progression (measured as a decrease of M_2 receptor density on cholinergic fibres) might be possible. If no way is found to directly label **(+)-72B** as a PET tracer, further studies with fluorine congeners are needed to find a suitable molecular structure, combining binding characteristics of **(+)-72B** with a good possibility for PET-labelling.

6 Summary

6.1 General considerations

GPCRs and ligand-gated ion channels mediate a great variety of physiological effects within the human brain and periphery. The search for selective ligands at these target sites as pharmacological tools or new drug candidates is of great interest. With increasing knowledge of the great diversity of some receptor families, compounds formerly considered to be selective, turned out to be non-selective with regard to recently identified subtypes, splice variants or additional receptor subunits. This work provides SAR studies by means of radioligand binding experiments at serotonergic h5-HT_{3A} and h5-$HT_{4(b)}$ receptors, histamine hH_1 receptors and muscarinic $hM_{1\text{-}5}$ receptors.

6.2 Compounds related to ondansetron

Ondansetron (**1**) is well known as a highly potent 5-HT_3 antagonist and served as lead structure for the development of other 5-HT_3 receptor antagonists. In our study a series of ondansetron congeners (**2** - **18**) with modification in the imidazolyl part or the side chain were investigated at recombinant h5-HT_{3A} receptors and data were compared to preliminary functional studies at native 5-HT_3 receptors in guinea-pig ileum. It could be clearly demonstrated within a series of regioisomers (**2A/B** - **6A/B**) that compounds with substituents at position 4 of the imidazole moiety displayed higher affinities than their isomers with substituent in position 5. An optimum within our series was the 4-methyl substituted congener **2A** displaying the highest measured affinity value (pK_i = 9.05) equaling a 2-fold increase in comparison to the parent compound. Expanding the size of the substituents led to decreased affinities. Side chain length had an optimum with n=2. Pronounced stereoselectivity was determined for the enantiomers **(+/-)-14** with a stereoselectivity ratio of 26. Comparison of functional data in guinea-pig ileum with binding studies at h5-HT_{3A} receptors revealed a low correlation and confirmed these receptors to be true species orthologues. Consequently, further studies in the drug development process of 5-HT_3 ligands should not use guinea-pigs as laboratory animals, because the obtained results have only little predictive power for human therapy.

6.3 Compounds related to metoclopramide

The prokinetic actions of the benzamide metoclopramide (MCP) were discovered long before 5-HT_4 receptors were described for the first time. In our study, we examined a series of compounds (**20** - **30**) related to the MCP ester congener SDZ205-557 (**19**) regarding their affinities to h5-$HT_{4(b)}$ receptors. The obtained binding data were compared with functional data derived in experiments at native 5-HT_4 receptors in guinea-pig ileum and rat oesophagus. Insertion of substituents in the side chain decreased binding affinity enormously and was not tolerated (**20**, **21**). Exchange of the amino moiety into a piperidine ring (**22**) led to a 4-fold increased affinity. A stereoselectivity ratio of 8 was calculated for the p-methyl substituted piperidine congeners **(+/-)-23**. In contrast to the 5-HT_{3A} subtype, bulky substituents in p-position of the basic ring were tolerated well and highest affinities within this series was found for compound **(±)-26** (pK_i = 8.43). Side chain length had an optimum with n=2. In contrast to 5-HT_3 receptors, binding data correlated well with functional data obtained at rat and guinea-pig 5-HT_4 receptors, suggesting these receptors to be species homologues.

6.4 Analogues of McN-A-343

Congeners of McN-A-343 (**31**) were extensively studied as ligands at muscarinic receptors. In our studies we examined a series of analogues (**32** - **42**) for their binding affinities to 5-HT_{3A}, 5-$HT_{4(b)}$ and H_1 receptors. At the 5-HT_{3A} subtype, compound **(R)-33$^+$** had the highest affinity among the tested compounds (pK_i = 5.74). Very low affinities were detected at the 5-$HT_{4(b)}$ subtype. All compounds were less potent than the parent compound **31** (pK_i = 5.73). At both subtypes, no preference was found for tertiary compounds over their N-methylated, quaternary congeners. More interesting findings turned out at H_1 receptors. Introduction of substituents within the chain had an optimum at a 4-F-phenyl substituent **(±)-38** (pK_i = 6.98), which displayed the highest affinity within this series. Tertiary compounds had higher affinities than their quaternary congeners. For six pairs of enantiomers (**33**, **33$^+$**, **36**, **36$^+$**, **37**, **37$^+$**), pronounced inverse stereoselectivity was found between M_1 and H_1 receptors. This was most pronounced for compound **(R/S)-37** with stereoselectivity ratios of 1660 and 0.09 at M_1 and H_1, respectively. Inverse stereoselectivity between

M_1 and H_1 receptors was reported for several chemically different compounds and is perhaps more common as generally assumed. Resolution of well known racemic H_1 antagonists into pure enantiomers might provide new starting points as novel lead structures in the search for selective muscarinic antagonists.

6.5 Compounds related to glycopyrrolate

A mixture of the enantiomers (3S, 2'R)- and (3R, 2'S)-glycopyrronium, named glycopyrrolate (Robinul®), is approved to the market and in therapeutic use as a parasympatholytic drug for premedication prior to surgical operations. Glycopyrronium (**43**) exists in 4 stereoisomers **43A-D**, which were investigated in preliminary studies in our laboratory. In this study, we investigated a series of congeners (**44** - **56**) for affinity to $M_{1\text{-}5}$ receptors. Exchange of a methyl substituent at the quaternary nitrogen by other groups, **Dia-44D** - **Dia-48D**, left affinities unchanged. Compounds **49A-D**, with a chinuclidine system instead of a pyrrolidine ring, displayed a stereochemical behaviour at $M_{1\text{-}5}$ receptors comparable to the parent compound. The (2'S)-configurated compounds **49A** and **49B** had lower affinities in comparison to the (3R, 2'R)-configurated compound **49D** (K. Kreutzmann, personal communication), whereas only minor losses in affinities were found for the (3S, 2'R)-configurated isomer **49C**. The highest affinities within this series were measured for **51D** (pK_i = 10.71 at M_2). Hybrid molecules, consisting of parts of glycopyrronium and tiotropium, **(R)-55** and **(R)-56**, were potent antagonists, too. Most tested compounds had very high affinities to muscarinic receptor subtypes, but displayed no selectivity for a single subtype in equilibrium binding studies.

6.6 Characterisation of [^{3}H](3R, 2'R)-glycoyrronium

Preliminary studies in our laboratory revealed the (3R, 2'R)-configurated enantiomer **43D** to possess kinetic selectivity for M_3 versus the M_2 receptors, based on pronounced differences in dissociation half-lifes at these subtypes. In this study, the most interesting compound (3R, 2'R)-glycopyrronium (**43D**) was labelled radioactively and completely characterised at $M_{1\text{-}5}$ receptors. [^{3}H]43D was found to be a highly potent, non-selective compound in competition and saturation

experiments (pK_D = 9.67 - 10.55), recognising the same receptor site as the standard radioligand [^{3}H]NMS. Results of kinetic studies with [^{3}H]43D confirmed the findings with the cold drug at M_2 and M_3 receptors and proved a kinetic selectivity for the M_3 subtype. Dissociation half-lifes at $M_{1\text{-}5}$ were 71, 23, 145, 77 and 100 min, respectively. Preclinical *in vivo* data demonstrated superior bronchodilator effects and prolonged time of action in comparison to ipratropium. Planned clinical studies with inhaled **43D** for use in COPD will elucidate whether a once- or a twice-daily dosing regimen will be suitable for human therapy. **43D** is to our knowledge the first compound following tiotropium with kinetic selectivity for M_3 receptors and is a promising candidate for COPD therapy in humans.

6.7 M_2-selective antagonists related to dimethindene

Since the discovery of inverse stereoselectiviy between muscarinic receptors and the H_1 subtype for the enantiomers of dimethindene (**57A**), studies in our laboratories were carried out in order to develop M_2-selective antagonists related to **57A**. It turned out to be difficult to obtain compounds with high affinity to the M_2 subtype and at the same time to reach high selectivity and specificity. In this study, we examined several analogues (**57B** and **58** - **83**) with special focus on side chain length and substitution pattern of the basic amino moiety. It was demonstrated that compounds with reduced chain length had increased affinities at the M_2 subtype and at the same time decreased H_1 affinities in most cases. As an optimum for the substitution pattern at the basic amino moiety an isopropyl group and a phenylethyl substituent were found in **(+)-72B**. This compound is a major break-through in our studies, combining high affinity to M_2 receptors (pK_i = 8.59) with good selectivity (M_2 versus $M_{1,3,4,5}$ 79-fold, 102-fold, 31-fold, 120-fold, respectively) and specificity versus H_1 receptors (1349-fold). **(+)-72B** is one of the most selective M_2 antagonists known today. **(+)-72B** is a potential candidate as a PET-ligand for diagnostic use in AD or as a therapeutic drug in AD.

7 Zusammenfassung

7.1 Generelle Aspekte

G-protein-gekoppelte Rezeptoren und Liganden-gesteuerte Ionenkanäle vermitteln eine Vielzahl physiologischer Effekte im menschlichen Gehirn und der Körperperipherie. Selektive Liganden an diesen Rezeptoren sind nicht nur als pharmakologische Werkzeuge, sondern auch als Arzneistoffkandidaten von Interesse. Mit zunehmendem Wissen über die Komplexität einzelner Rezeptorfamilien stellte sich heraus, dass Stoffe, die zuvor als selektive Liganden angesehen wurden, nicht zwischen den altbekannten und neu entdeckten Subtypen, Splicevarianten oder Untereinheiten unterscheiden können. Diese Arbeit stellt einen Beitrag auf dem Gebiet der Struktur-Wirkungs Beziehungen an muskarinischen, serotonergen und histaminergen Rezeptoren mittels Radioligandbindungsstudien dar.

7.2 Analoga des Ondansetrons

Ondansetron (**1**) hat als hochpotenter 5-HT_3 Antagonist bei der Entwicklung anderer 5-HT_3 Blocker als Leitstruktur gedient. In unserer Studie wurden Analoga des Ondansetrons (**2** - **18**) mit Modifikationen im Imidazolring oder der Seitenkette untersucht und mit vorherigen Ergebnissen aus funktionellen 5-HT_3 Studien am Meerschweinchen-Ileum verglichen. In einer Reihe von 4/5-Stellungsisomeren (**2A/B** - **6A/B**) konnte eindeutig gezeigt werden, dass die Verbindungen mit Substituenten in 4-Position höhere Affinitäten aufwiesen als ihre Stellungsisomere. Die höchste Affinität innerhalb dieser Serie wurde für die 4-Methyl-substituierte Verbindung **2A** gemessen (pK_i = 9.05), die 2-fach potenter als die Muttersubstanz war. Größere Substituenten führten zu verringerter Affinität. Eine Kettenlänge von n=2 wurde als Optimum bestimmt. Hohe Stereoselektivität wurden für die Enantiomere **(+/-)-14** mit einem Quotienten von 26 bestimmt. Ein Vergleich der Bindungsdaten am h5-HT_{3A} Rezeptor mit den funktionellen Daten am Meerschweinchen-Ileum, zeigte eine schlechte Korrelation. Die 5-HT_3 Rezeptoren von Mensch und Meerschweinchen können als echte Spezies-Orthologe betrachtet werden. Als Ergebnis dieser Studie sollte bei der weiteren Entwicklung von 5-HT_3 Liganden das Meerschweinchen als Labortier vermieden werden, da die so

ermittelten Werte von nur geringer Vorhersagekraft für die Therapie beim Menschen sind.

7.3 Analoga des Metoclopramids

Die prokinetischen Eigenschaften des Benzamids Metoclopramid (MCP) wurden lange vor der ersten Beschreibung von 5-HT_4 Rezeptoren entdeckt. In unserer Studie wurde eine Serie von Derivaten (**20** - **30**) des MCP Ester-Analogons SDZ205-557 (**19**) auf ihre Bindungseigenschaften an h5-$HT_{4(b)}$ Rezeptoren untersucht und mit funktionellen 5-HT_4 Daten am Ileum des Meerschweinchens und der Speiseröhre der Ratte verglichen. Das Einfügen von Substituenten in der Seitenkette (**20**, **21**) wurde nicht toleriert und führte zu einer deutlichen Affinitätsabnahme. Das Einbeziehen des basischen Stickstoffs in ein Piperidinringsystem (**22**) führte zu einem 4-fachen Anstieg der Affinität. Ein Stereoselektivitätsquotient von 8 wurde für die p-Methyl-substituierten Piperidinderivate **(+/-)-23** bestimmt. Im Gegensatz zum 5-HT_{3A} Rezeptor wurden große Substituenten in p-Position toleriert. Der höchste Wert innerhalb dieser Gruppe wurde für **(±)-26** (pK_i= 8.43) bestimmt. Eine Kettenlänge von n=2 wurde als Optimum gefunden. Im Gegensatz zum 5-HT_3 Rezeptor wurde beim Vergleich der Bindungsdaten mit den funktionellen Daten von Ratte und Meerschweinchen am 5-HT_4 Rezeptor eine gute Korrelation gefunden. Die Rezeptoren können als Spezies-Homologe betrachtet werden.

7.4 Analoga des McN-A-343

Verwandte des McN-A-343 (**31**) wurden ausgiebig auf ihre Affinität zu muskarinischen ACh Rezeptoren untersucht. In dieser Studie wurde eine Gruppe von Derivaten (**32** - **42**) auf ihre Affinitäten an 5-HT_{3A}, 5-$HT_{4(b)}$ und H_1 Rezeptoren untersucht. An 5-HT_3 Rezeptoren hatte **(R)-33$^+$** die höchste Affinität aller untersuchten Substanzen (pK_i =5.74). Sehr geringe Affinitäten wurden am 5-HT_4 Rezeptor gemessen, wobei für die Muttersubstanz **31** der höchste Wert bestimmt wurde (pK_i = 5.73). Bei beiden Rezeptoren wurde keine Präferenz für tertiäre oder quartäre Verbindungen festgestellt. Interessantere Ergebnisse wurden am H_1 Rezeptor gefunden. Die Einführung eines Restes in der Kette zeigte ein Optimum bei

(±)-38 (pK_i = 6.98) mit einen 4-F-Phenylrest. Die tertiären Verbindungen waren stets potenter als ihre quartären, N-Methylierten Analoga. Für sechs Enantiomerenpaare (**33**, **33$^+$**, **36**, **36$^+$**, **37**, **37$^+$**) wurde eine inverse Stereoselektivität zwischen M_1 und H_1 Rezeptoren beobachtet. Am stärksten war dieser Effekt bei **(R/S)-37** ausgeprägt, wo Stereoselektivitätsquotienten von 1660 and 0.09 für M_1 bzw. H_1 bestimmt wurden. Inverse Stereoselektivität zwischen muskarinischen und Histamin H_1 Rezeptoren wurde schon für strukturell verschiedene Moleküle beobachtet und ist vielleicht weiter verbreitet als generell angenommen wird. Das Auftrennen von bekannten, racemischen H_1 Antagonisten in reine Enantiomere könnte neue Ausgangpunkte für Leitstrukturen auf der Suche nach selektiven muskarinischen Antagonisten liefern.

7.5 Analoga des Glycopyrroniums

Derzeit ist ein Gemisch der Enantiomere (3S, 2'R)- und (3R, 2'S)-Glycopyrronium als Parasympatholytikum zur Prämedikation vor chirurgischen Eingriffen auf dem Markt zugelassen (Robinul®). Glycopyrronium (**43**) existiert in vier verschiedenen Stereoisomeren **43A-D**, die in vorangegangen Studien in unserem Labor untersucht wurden. In der vorliegeden Studie wurden eine Reihe verwandter Substanzen (**44** - **56**) auf ihre Affinität zu muskarinischen Rezeptoren untersucht. Der Austausch eines Methylsubstituenten am quartären Stickstoff durch andere Reste führte bei **Dia-44D** - **Dia-48D** zu keiner nennenswerten Veränderung der Affinitäten. Die Verbindungen **49A-D**, mit einem Chinuclidin-System anstelle des Pyrrolidinrings der Muttersubstanz, zeigten das gleiche stereochemische Verhalten wie die Muttersubstanz. Die (2'S)-konfigurierten Stereoisomere **49A** und **49B** hatten drastisch geringere Affinitäten im Vergleich zur (3R, 2'R)-konfigurierten Verbindung **49D** (K. Kreutmann, persönliche Mitteilung), wohingegen nur eine geringe Abnahme der Affinität beim (3S, 2'R)-konfigurierten Stereoisomer **49C** beobachtet wurde. Die höchsten Affinitäten innerhalb dieser Serie wurde für **51D** (pK_i = 10.71 an M_2) bestimmt. Auch die Tiotropium-Glycopyrronium-Hybrid Moleküle **(R)-55** und **(R)-56** wiesen sehr hohe Affinitäten auf. Die meisten der untersuchten Verbindungen waren hochpotente, muskarinische Antagonisten, jedoch zeigten sie keinerlei Selektivität in Bindungsexperimenten.

7.6 Charakterisierung von [^{3}H](3R, 2'R)-Glycopyrronium

Vorangegangene Studien in unserer Arbeitsgruppe hatten gezeigt, dass das (3R, 2'R)-konfigurierte Isomer des Glycopyrroniums (**43D**) kinetische Selektivität für M_3 gegenüber M_2 Rezeptoren aufweist, die auf unterschiedlichen Dissoziationshalbwertszeiten an diesen Subtypen beruht. In der vorliegenden Studie wurde diese Verbindung radioaktiv markiert und komplett an M_{1-5} Rezeptoren charakterisiert. Dabei erwies sich [^{3}H]43D als hochpotenter, unselektiver Antagonist in Verdränguns- und Sättigungsexperimenten (pK_D = 9.67 - 10.55), wo er an die gleiche Bindungsstelle angriff wie der Referenz-Radioligand [^{3}H]NMS. Die Ergebnisse der kinetischen Untersuchungen bestätigten die Verhältnisse, die mit der nicht markierten Verbindung an M_2 und M_3 Rezeptoren gefunden worden waren und belegten eine kinetische Selektiviät für den M_3 Rezeptor. Die berechneten Dissoziationshalbwertszeiten betrugen 71, 23, 145, 77 und 100 min an M_{1-5} Rezeptoren. Präklinische *in vivo* Daten zeigten eine überlegene Bronchodilatation und eine verlängerte Wirkdauer für **43D** im Vergleich zu Ipratropium. Die geplanten klinischen Studien zur Therapie der COPD mit inhaliertem **43D** werden zeigen, ob eine 2x tägliche Gabe oder sogar eine Einmalgabe zur Therapie beim Menschen ausreichend sind. Nach unserem Wissen ist **43D** die erste Substanz nach Tiotropium mit kinetischer Selektivät für M_3 Rezeptoren und ist ein vielversprechender Kandidat für die COPD Therapie beim Menschen.

7.7 M_2-selektive Antagonisten abgeleitet von Dimethinden

Nach der Entdeckung der inversen Stereoselektivität zwischen muskarinischen Rezeptoren und dem H_1 Subtyp bei den Enantiomeren des Dimethindens (**57A**), wurden in unseren Labors Untersuchungen durchgeführt, mit dem Ziel, einen hochaffinen M_2-selektiven Antagonisten ausgehend von **57A** zu entwickeln. Es stellte sich jedoch als äußerst schwierig heraus, eine Substanz zu finden, die eine hohe Affinität zum M_2 Rezeptor in Kombination mit einer guten Selektivität und Spezifität aufwies. In unserer Studie wurde eine Reihe von Derivaten (**57B** und **58** - **83**) mit unterschiedlicher Länge der Seitenkette und verändertem Substitutionsmuster an der Aminogruppe untersucht. Es konnte gezeigt werden, dass Verbindungen mit verkürzter Seitenkette eine erhöhte Affinität zum M_2 Rezeptor aufwiesen und

gleichzeitig in den meisten Fällen eine verringerte Affinität zum H_1 Subtyp zeigten. Als optimale Substituenten an der Aminogruppe wurden ein Isopropyl- und ein Phenylethylrest bei **(+)-72B** identifiziert. Diese Verbindung stellt einen großen Durchbruch dar, da sie eine hohe Affinität zu M_2 (pK_i = 8.59) mit guter Selektivität (M_2 versus $M_{1,3,4,5}$ 79-fach, 102-fach, 31-fach, 120-fach) und Spezifität gegenüber H_1 (1349-fach) vereint. **(+)-72B** ist einer der selektivsten derzeit bekannten M_2 Antagonisten. **(+)-72B** stellt einen hoffnungsvollen Kandidaten, zur Entwicklung eines PET-Liganden zur Diagnose der Alzheimer Erkrankung dar und ist darüber hinaus ein potentielles Arzneimittel zur Therapie dieser Krankheit.

8 Abbreviations

AC	Adenylate cyclase
ACh	Acetylcholine
AChE	Acetylcholine esterase
AD	Alzheimer's disease
AF-DX116	11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepin-6-one
AF-DX384	5,11-Dihydro-11-[[[2-[2-(dipropylamino)methyl)-1-piperidinyl]ethyl]amino]carbonyl]-6H-pyrido(2,3-b) (1,4)benzodiazepin-6-one
APP	Amyloid precursor protein
sAPP	Soluble APP
BIBN99	5,11-Dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1-oxopentyl) ethylamino]propyl]-1-piperidinyl]acetyl]-6H-pyrido(2,3-b) (1,4)benzodiazepine-6-one
BIMU1	Endo-N-(8-methyl-8-azabicyclo[3.2.1]-oct-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazol-1 carboxamine
cAMP	Cyclic adenosine-3',5'-monophosphate
ChAT	Choline acetyletransferase
CHO-K1 cells	Chinese hamster ovary cells
CPM	Counts per minute
COPD	Chronic obstructive pulmonary disease
DAG	Diacylglycerol
4-DAMP	4-Diphenylacetoxy-N-methylpiperidinium methoiodide
GPCR	G-protein coupled receptor
GPI	Guinea-pig ileum
G-protein	Guanine nucleotide binding protein
GR113808	1-methyl-1H-indole-3-carboxylic acid, [1-[2-[(methyl-sulfonyl)amino]ethyl]-4-piperidinyl]methyl ester
HEK293 cells	Human embryonic kidney cells
HHSiD	Hexahydro-sila-difenidol
5-HT	5-Hydroxytryptamine, Serotonin
IBS	Irritable bowel syndrome

IC_{50}	Concentration of an antagonist producing halfmaximal inhibition
IP_3	Inositol-1,4,5-triphosphate
IOP	Intraocular pressure
IUPHAR	International Union of Pharmacology
K_D	Equilibrium dissociation constant
$kinK_D$	K_D value determined in kinetic binding experiments
$satK_D$	K_D value determined in saturation binding experiments
K_i	Inhibition constant (equilibrium dissociation constant of the inhibitor)
KO	Knock out
k_{on}	Association rate constant
k_{off}	Dissociation rate constant
McN-A-343	4-(3-Chlorophenylcarbamoyloxy)-2-butynyltrimethyl-ammonium chloride
MCP	Metoclopramide
MDL72222 (bemesetron)	Tropanyl 3,5-dichlorobenzoate
n	Number of individual experiments
NMS	N-methyl scopolamine
PET	Positron emission tomography
PD	Parkinson's disease
PD102807	9-Methoxy-2-methyl-11,12-dihydro-3H,6αH,13H-6-oxa-3,12α-diaza-benzo[a]cyclopenta(h)anthracene-1-carboxylic acid ethyl ester
PD150714	1-Ethyl-4-phenyl-1,2,3,6-tetrahydropyridine-3-carboxylic acid hexyl ester ethanediolate
PKA	Proteinkinase A
PLC	Phospholipase C
ROS	Rat oesophagus
RS23597-190	3-(Piperidin-1-yl)propyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride

RS39604	1-[4-Amino-5-chloro-2-(3,5-dimethoxyphenyl)methyloxy]-3-[1-[2-methylsulphonylamino]ethyl]piperidin-4-yl]propan-1-one hydrochloride
SAR	Structure-activity relationship
SB203186	1-Piperidinylethyl-1H-indole-3-carboxylate hydrochloride
SCH57790	4-Cyclohexyl-α-[4-[4-methoxyphenyl]-(S)-sulfinyl]-phenyl]-1-piperazineacetonitrile
SD	Standard deviation
SR57227A	4-Amino-(6-chloro-2-pyridyl)-1 piperidine hydrochloride
TM	Transmembrane domain

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