Allosteric modulation by sodium ions and amilorides of G protein-coupled receptors : a closer look at the sodium ion site of the adenosine A2a receptor and development of a mass spectrometry ligand binding assay for adenosine A1 and A2a receptors

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Chapter 2

Allosteric modulation of class A GPCRs by amiloride and its analogues

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Abstract

The function of G protein-coupled receptors (GPCRs) can be modulated by compounds that bind to other binding sites than the endogenous orthosteric site, called allosteric sites. Recent crystal structures revealed a bound sodium ion in a conserved allosteric site of multiple GPCRs. Amiloride and its analogues have been proposed to bind in this same sodium ion site. This review seeks to recapitulate the current knowledge of allosteric effects by amilorides and its analogues on class A GPCRs. Amilorides are known to modulate adenosine, adrenergic, dopamine, chemokine, muscarinic, serotonin and gonadotropin-releasing hormone receptors. Amiloride analogues with lipophilic substituents are more potent modulators than amiloride for several GPCRs. Some receptors, like adenosine, α-adrenergic, and dopamine receptors, are strongly modulated by amiloride analogues. In addition, for a few GPCRs more than one binding site for amilorides has been postulated. Interestingly, the allosteric effects by amiloride binding vary considerably between GPCRs, from acting as negative allosteric modulators to in some cases as positive allosteric modulators. Since the sodium ion site is strongly conserved amongst GPCRs it is to be expected that amilorides also bind to GPCRs not yet evaluated for their sensitivity, but with different modulating effects. Investigating this typical amiloride-GPCR interaction further may yield general insight on the allosteric mechanisms of GPCR binding and function.

Introduction

G protein-coupled receptors (GPCRs) form a family of receptors with approximately 800 members that are responsible for many different physiological functions such as regulation of sleep, vision, blood pressure, CNS activity, taste, and olfaction.[1] This is reflected by the fact that they are targeted by 30% to 40% of therapeutic drugs currently on the market.[2] GPCRs are grouped according to their structural and genomic characteristics in five main groups: rhodopsin-like (class A), secretin-like (class B), glutamate-like (class C), adhesion, and frizzled/taste2, with class A being the largest group.[3, 4]

The precise mechanisms of these receptors have been studied for a long time, but due to the complexity of their structures they are not yet fully understood. The recent increase in availability of high resolution GPCR crystal structures allow a better understanding of how GPCRs function.[5, 6] Co-crystallization with orthosteric ligands allows the study of endogenous orthosteric binding sites. However, to study allosteric binding sites co-crystallization with allosteric modulators is necessary, which is a challenge due to their relatively low affinities. Adding high concentrations of sodium ions is a common procedure in the crystallization of GPCRs to stabilize the protein, which makes it possible for them to bind to even low affinity sites. However, sodium ions are relatively small and will need a high resolution (< 2 Å) to be visualized. In recent crystal structures of several GPCRs the resolution was sufficiently high to locate a sodium ion bound in a site which is highly conserved amongst GPCRs.[7] Currently solved crystal structures with a sodium ion bound in its allosteric site are of the human adenosine A_{2A} receptor,[8] the β_1-adrenergic receptor,[9, 10] the human δ-opioid receptor,[11] and the human protease activated receptor 1.[12] The common residues that interact with the sodium ion in these crystal structures, either directly or through water mediated hydrogen bond interactions, are $Asp^{2.50}$, $Ser^{3.39}$ $Trp^{6.48}$, $Asn^{7.45}$, and $Asn^{7.49}$ (numbering according to Ballesteros-Weinstein[13]). The negatively charged amino acid $Asp^{2.50}$ makes a strong salt bridge with the positively charged sodium ion and is essential for its binding in this site, which confirmed previous 'pre-crystal structure' research.[14] It is also the most conserved residue of the sodium ion site amongst GPCRs. The high conservation of the sodium ion pocket amongst GPCRs makes it probable that more crystal structures with sodium ions bound in this site are to be expected.

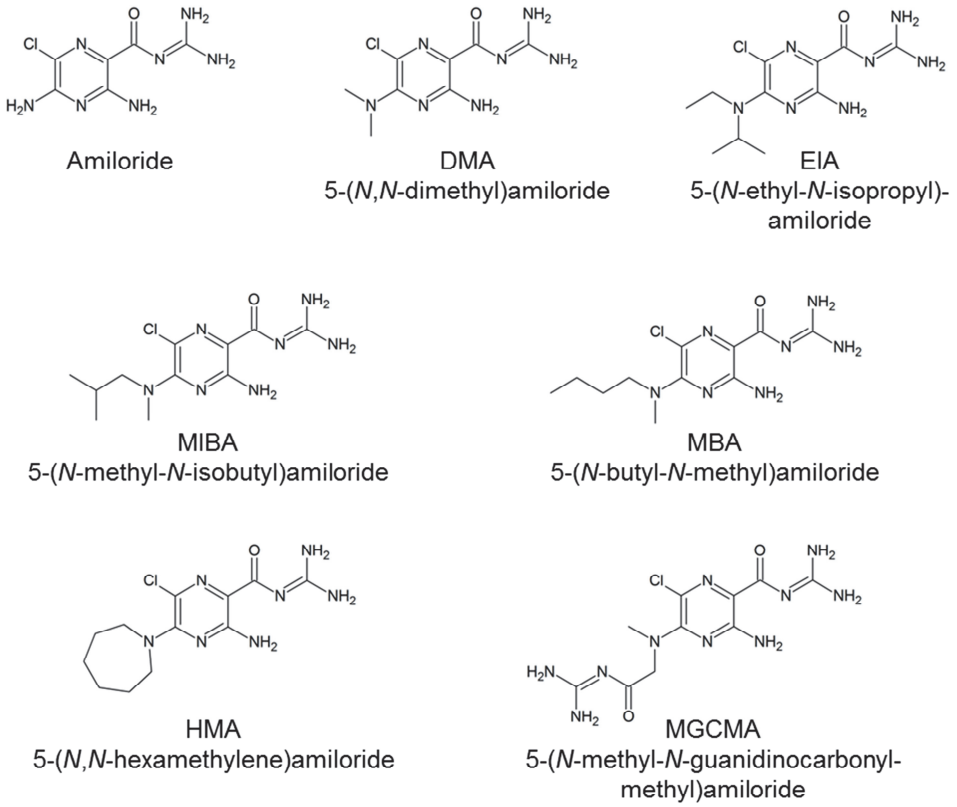

Figure 1. Chemical structures of amiloride and its 5'-amino substituted analogues DMA, EIA, MIBA, MBA, HMA, and MGCMA.

Amiloride is primarily known as a potassium sparing diuretic drug, acting through the blockade of renal epithelial sodium channels.[15] Amiloride and its analogues have also been found to bind to the sodium ion site of several GPCRs, modulating orthosteric ligand binding.[16] The negatively charged carboxylate of sodium ion site residue $Asp^{2.50}$ interacts with the positively charged guanidinium group present on all amilorides, which makes it important for amiloride binding as discussed in Chapters 3 and 4 of this thesis. The binding of amilorides into the sodium ion site of GPCRs renders these compounds potential pharmacological tools to probe molecular mechanisms of GPCR allosteric modulation. This review summarizes the current knowledge of modulation by amiloride and its analogues on class A GPCRs, which primarily occurs through binding in the sodium ion site. The chemical structures of amiloride and its analogues discussed in this review are depicted in Figures 1 and 2. Effects of the amilorides are represented in Table 1 categorized per GPCR

and orthosteric ligands used. Most of the receptors in Table 1 are discussed in the main text, except for the histamine H_1 and the opioid receptors which have been studied little for amiloride effects.

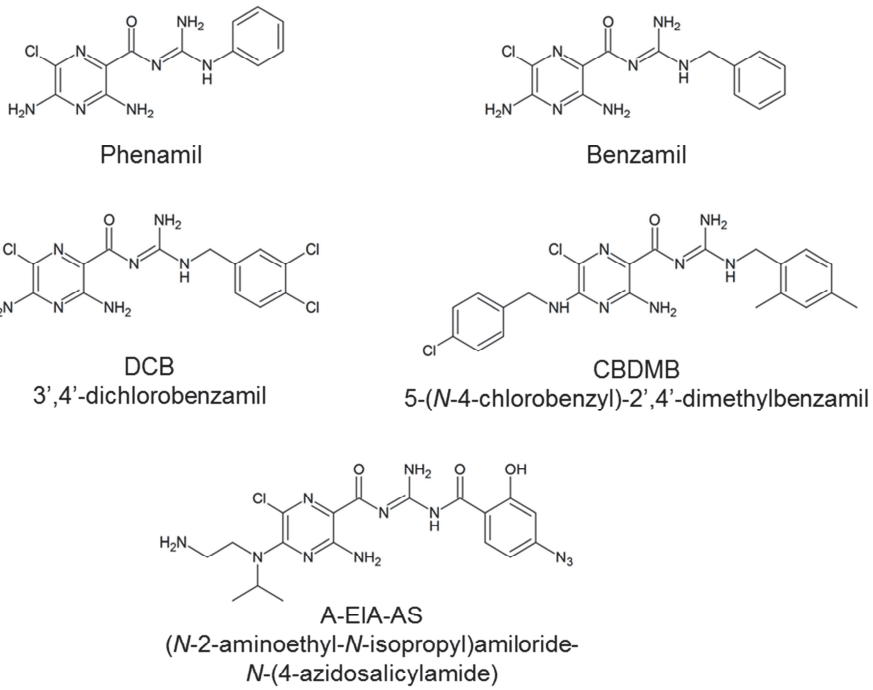

Figure 2. Chemical structures of 2-guanidino substituted amiloride analogues phenamil, benzamil, DCB, CBDMB, and A-EIA-AS.

Adenosine receptors

Adenosine receptors have been studied extensively, and as a result many orthosteric[17] and allosteric[18] ligands have been discovered. Amiloride interactions with adenosine receptors were discovered in the early days of adenosine receptor research.[19] Since the effects of amiloride binding to adenosine receptors appeared to be closely tied to sodium ion interactions, it was necessary to investigate and exclude the involvement of Na^+/H^+ exchangers (one of the main targets of amiloride) in these interactions.[16] In this study, Garritsen et al. found inhibition of antagonist [^{3}H]DPCPX and agonist [^{3}H]PIA at the calf adenosine A_1 receptor by amiloride, its 5'-amino substituted analogues 5-(*N*,*N*-

hexamethylene)amiloride (HMA), 5-(*N*-methyl-*N*-butyl)amiloride (MBA), 5-(*N*-methyl-*N*-guanidinocarbonyl-methyl)amiloride (MCGMA), and 5-(*N*-methyl-*N*-isobutyl)amiloride (MIBA), and its 2-guanidino substituted analogues benzamil, 5-(*N*-4-chlorobenzyl)-2',4'-dimethylbenzamil (CBDMB), 3',4'-dichlorobenzamil (DCB), and phenamil.

Gao and IJzerman found that amiloride analogues benzamil, HMA, MCGMA, MIBA, and phenamil increased the dissociation rate of the antagonist [^{3}H]ZM-241,385 at the rat A_{2A} receptor, and that they were more potent than amiloride itself (Figure 3).[20] However, the affinity (defined by radioligand displacement in equilibrium) and the allosteric potency (defined by the concentration-dependent effect on the radioligand dissociation rate) did not correlate. This indicated a mixed competitive (i.e., mutually exclusive displacement) and noncompetitive (i.e., amilorides and orthosteric ligands can bind to the receptor at the same time, and amiloride influences the orthosteric ligand's dissociation rate) behavior of amilorides. The amiloride analogues HMA and MIBA, with a lipophilic moiety on the 5'-position, proved to be the most potent compounds in increasing the dissocation rate of the orthosteric ligand, while they had equal affinities to benzamil and phenamil in displacing it. In contrast to the effect of amilorides, sodium ions decreased the dissociation rate of [^{3}H]ZM-241,385. Still, sodium ions and HMA appeared to compete for the same allosteric site.

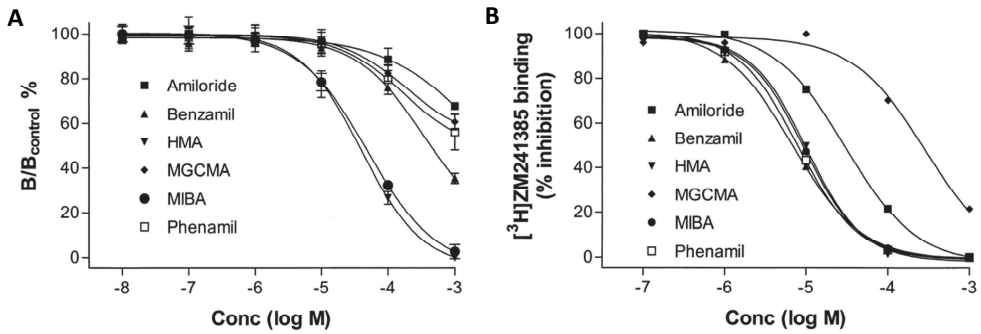

Figure 3. Concentration dependence of amiloride and its analogues for increase of [^{3}H]ZM-241,385 dissociation **(A)** and displacement of [^{3}H]ZM-241,385 **(B)** after reaching binding equilibrium at adenosine A_{2A} receptors. In **A** [^{3}H]ZM-241,385 was allowed to first reach equilibrium binding at the receptor before the dissociation was induced by addition of an excess of antagonist, in absence and presence of increasing concentrations of amiloride (analogue). The results are expressed as a ratio between the binding of [^{3}H]ZM-241,385 after 120 min in presence ('B') and in absence ($B_{control}$) of amiloride (analogue). Reproduced with permission from Gao and IJzerman.[20]

In a study by Gao et al. in 2003 it appeared that agonists and antagonists are differently affected by amilorides on adenosine receptors.[21] Amilorides increased the dissociation rates of antagonists [^{3}H]DPCPX at the rat adenosine A_1 and [^{3}H]PSB-11 at the human A_3 receptors, just as with [^{3}H]ZM-241,385 at the rat A_{2A} receptor. However, they did not affect the dissociation rates of agonists [^{3}H]*R*-PIA from the rat A_1 and [^{3}H]CGS-21,680 from the rat A_{2A} receptors. At the A_{2A} receptor amilorides still displaced agonist [^{3}H]NECA binding in a mutually exclusive manner, which makes them competitive inhibitors of agonist binding (Chapter 3). Amilorides even decreased the dissociation rate of agonist [^{125}I]-AB-MECA at the rat adenosine A_3 receptor, revealing that amilorides can also act as positive allosteric modulators depending on the radiolabeled probe used.[21] Furthermore the amilorides exhibited selectivity for the different adenosine receptor subtypes. Amiloride and 5-(*N*,*N*-dimethyl)amiloride (DMA) were more potent at the A_1 receptor in accelerating antagonist dissociation, while HMA was the most potent at the A_{2A} receptor and to a lesser extent at the A_3 receptor.

Solving the crystal structure of the adenosine A_{2A} receptor at a resolution of 1.8 Å provided a sufficiently high resolution to detect for the first time a sodium ion bound in its allosteric binding site.[8] The amino acids interacting with the sodium ion are highly conserved amongst GPCRs which confirmed previous studies in which modulation by sodium ions was tied to the same amino acids for different GPCRs.[7] The most conserved amino acid is a negatively charged aspartic acid (Asp52$^{2.50}$) which interacts directly with the positively charged sodium ion by means of a salt bridge. The positively charged guanidinium moiety of amilorides may also interact in a similar manner with this Asp52$^{2.50}$, and this is confirmed in Chapter 3 of this thesis by a docking study based on the A_{2A} crystal structure (Figure 4), and in Chapter 4 by a mutational study in which mutation of this aspartic acid into an alanine decreased the affinity of amiloride and HMA. In the same chapter, the mutation of other amino acids forming the sodium ion site, Trp246$^{6.48}$, Asn280$^{7.45}$, and Asn284$^{7.49}$, into Ala influenced the binding of amiloride and HMA as well. However, mutation of these residues increased the amilorides' affinity, especially in case of Trp246$^{6.48}$. At the adenosine A_3 receptor the mutation of Trp243$^{6.48}$ into Ala increased the affinity of HMA as well,[22] and it can be concluded that this residue strongly impedes the binding of amilorides in the sodium ion site of both receptors.

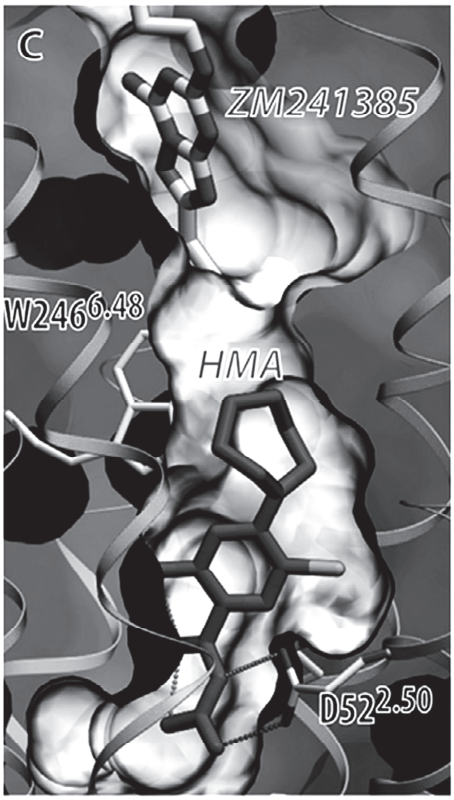

Figure 4. Docking of HMA in the sodium ion site. The guanidinium group of HMA has a salt bridge interaction with Asp52$^{2.50}$ whereas the 5'-azepane moiety of HMA clashes with Trp246$^{6.48}$. Chapter 3 provides more details about this docking study.

Adrenergic receptors

One of the first indications that amiloride inhibited the binding of orthosteric ligands at α- and β-adrenergic receptors was found in 1987 by Howard et al,[23] which was followed by many studies with amiloride and its analogues at adrenergic receptors. At the human α_{1A}-adrenergic receptor amiloride and its analogues benzamil, DMA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIA), MIBA, and HMA increased the dissociation rate of antagonist [^{3}H]prazosin, and the analogues with bulky lipophilic 5'-moieties were more potent in doing so.[24, 25] Amiloride itself was characterized as an allosteric modulator acting at one allosteric site, but all the amiloride analogues appeared to bind to two different allosteric sites. The authors speculated that these allosteric sites could be present on one receptor or were an effect of receptor dimerization, but could not further confirm this. The allosteric

interaction by amilorides was seemingly in contradiction with previous results at rat and mouse α_1-adrenergic receptors in which amiloride only showed a competitive interaction with antagonist [^{3}H]prazosin binding but did not influence its dissociation rate,[23] which may be attributed to the not yet discovered subtypes of α_1-receptors or the difference in species.

α_2-Adrenergic receptors are allosterically modulated by amilorides as well. At rat, human, bovine, and porcine α_{2A}-adrenergic receptors amiloride increased the dissociation rate of the antagonists [^{3}H]rauwolscine[23, 26] and [^{3}H]yohimbine.[27] Amiloride analogues also increased antagonist dissociation at the α_{2A}-adrenergic receptor, which was found for the amilorides (*N*-2-aminoethyl-*N*-isopropyl)amiloride-*N*-(4-azidosalicylamide) (A-EIA-AS) at the porcine receptor,[28] and DMA, EIA, MIBA, and HMA at the human receptor, in relation to [^{3}H]yohimbine, [^{3}H]rauwolscine, and [^{3}H]RX-821,002 dissociation.[29] It is noteworthy that A-EIA-AS has no affinity for the Na^+/H^+ exchange protein, making it a GPCR selective amiloride. EIA, HMA, and MIBA were exceptionally strong negative allosteric modulators of antagonist binding, being 50- to 80-fold more potent than amiloride in increasing the dissociation rate of [^{3}H]yohimbine, showing that bulky lipophilic moieties at the 5'-position of amiloride increase the allosteric potency at the α_{2A}-adrenergic receptor considerably. The apparent affinities of these amilorides were not correlating at all with their derived allosteric potencies in this study, cautioning to not confuse these two different pharmacological properties with each other.

In contrast to their effect on antagonists, amiloride, DMA, and HMA decreased the dissociation rate of agonist [^{3}H]UK-14,304 at the human α_{2A}-adrenergic receptor, with HMA having the largest effect.[30] The dissociation slowing effect on agonist binding (2.7-fold slower dissociation by HMA) was considerably smaller though than the dissociation accelerating effect on antagonist binding (140-fold faster dissociation by HMA). Even as they slowed agonist dissociation, amilorides acted as negative allosteric modulators of α_{2A} receptor agonist activation, since amiloride, DMA, and HMA decreased the potency of norepinephrine and UK-41,304. This paradoxical behavior was in line with previous indications that in addition to their allosteric effects amilorides could displace the orthosteric ligand competitively at the α_{2A} receptor.[29] Moreover, addition of sodium ions increased the affinity of amiloride in doing so.[23] This led to the conclusion that at α_{2A}-adrenergic receptors amilorides could bind to two different sites, namely the orthosteric

site and an allosteric sodium ion site. Howard et al. hypothesized that amiloride binding in the orthosteric site was enhanced by binding of a sodium ion in the allosteric site, while amiloride binding in the allosteric site increased the dissociation rate of other orthosteric ligands.[23] In a later study by Leppik et al., variations in the affinity of several amiloride analogues for the antagonist occupied and non-occupied receptor led to two different hypotheses. Either the amilorides could indeed bind to both the allosteric and orthosteric sites, or binding of an antagonist to the orthosteric site modified the conformation of the allosteric binding site in such a way that amiloride affinity decreased.[29]

At the α_{2B} subtype however, amilorides can both increase and decrease the dissociation rate of antagonists. The 5'-substituted amilorides EIA and MIBA increased the dissociation rate of [^{3}H]rauwolscine binding, while the guanidino-substituted amiloride CBDMB decreased it (Figure 5).[31]

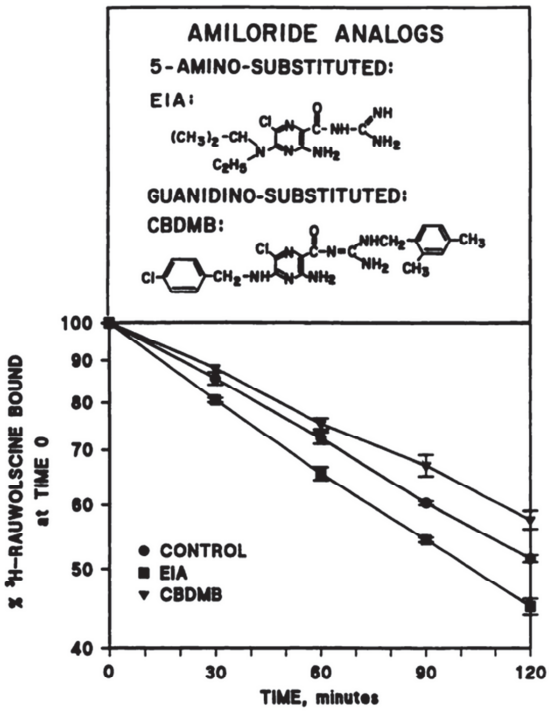

Figure 5. Positive and negative allosteric modulation of [^{3}H]rauwolscine binding at the α_{2B}-adrenergic receptor, in presence of 100 μM CBDMB and EIA, respectively. CBDMB decreased and EIA increased the dissociation rate of the orthosteric antagonist. Reproduced with permission from Wilson et al.[31]

The interaction of amiloride with the β-adrenergic receptors has only been studied by Howard et al. in 1987. At both the β_1- and β_2-adrenergic receptors amiloride displaced the antagonist [^{125}I]iodocyanopindolol competitively, since their binding was mutually exclusive.[23] Addition of sodium ions did not compete with amiloride binding, and it was concluded that amiloride did bind to the orthosteric site rather than to an allosteric sodium ion site. Even for the lack of modulation of β-adrenergic receptors by sodium ions and amiloride, a sodium ion site was found in the crystal structure of the β_1-adrenergic receptor.[9] The amino acids forming the sodium ion sites of the β_1-adrenergic and the adenosine A_{2A} receptor were the most similar of the solved GPCR crystal structures with such a site.[7] That makes the difference in modulation by sodium ions and amilorides between these receptors remarkable and it is probably due to differences in the overall architecture of the two receptors.

Chemokine receptors

Amiloride interactions with the chemokine receptor family have only been studied by Zweemer et al. on the chemokine CCR2 receptor.[32] The sodium ion site was the third binding site found on this receptor, next to the more extracellularly located orthosteric and an intracellular allosteric site.[33, 34] Amiloride analogues MIBA and HMA inhibited binding of antagonist [^{3}H]INCB3344 binding to the orthosteric site and antagonist [^{3}H]CCR2-RA-[R] binding to the intracellular site.[32] Moreover, HMA inhibited binding of the orthosteric agonist [^{125}I]CCL2. Amiloride, benzamil, MCGMA, and phenamil did however not displace any of these radioligands.

The increased dissociation rates of the orthosteric antagonist [^{3}H]INCB3344, the intracellular antagonist [^{3}H]CCR2-RA-[R], and the orthosteric agonist [^{125}I]CCL2 induced by HMA indicated a noncompetitive allosteric interaction. Remarkably, the dissociation rate of the agonist [^{125}I]CCL2 increased more (9.7-fold) than of the antagonists (1.25- and 1.36-fold) by the presence of HMA. Saturation binding assays revealed that HMA had a mixed competitive/noncompetitive interaction with the orthosteric antagonist [^{3}H]INCB3344 (insurmountable binding of HMA indicated by decreased B_{max} and competitive interaction indicated by increased K_D) while it had a purely noncompetitive interaction with the intracellular antagonist [^{3}H]CCR2-RA-[R] (decreased B_{max} only).

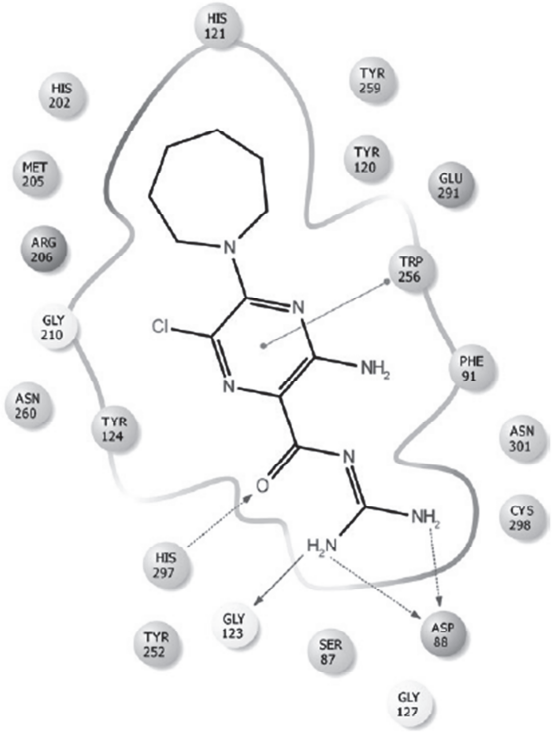

Figure 6. Two dimensional interaction map of HMA docking into the sodium ion pocket of a homology model of the CCR2 receptor, demonstrating a salt bridge between the guanidinium group of HMA and Asp88$^{2.50}$, π-stacking between the pyrazine core of HMA and Trp256$^{6.48}$, and a hydrogen bond between the oxygen of HMA and His297$^{7.45}$. The hydrogen bond between Trp256$^{6.48}$ and HMA's oxygen was not captured from this angle. Reproduced with permission from Zweemer et al.[32]

The allosteric effect of HMA was diminished by mutation of sodium ion site residues Asp88$^{2.50}$ and His297$^{7.45}$ into Ala. Mutation of Trp256$^{6.48}$ even completely abolished HMA's allosteric effect, which is in contrast to the observed increase of HMA's affinity by the same mutation in adenosine receptors as discussed by Gao et al.[22] and Chapter 4 of this thesis. Indeed, HMA fitted well in the sodium ion site of a homology model of the CCR2 receptor in a docking study, predicting interactions with these three residues (Figure 6). Asp88$^{2.50}$ formed a salt bridge with the guanidinium moiety of HMA, Trp256$^{6.48}$ formed a hydrogen bond with HMA's oxygen as well as engaging in a π-stacking interaction with its pyrazine core, and His297$^{7.45}$ formed a hydrogen bond with the same oxygen of HMA. Amino acid His297$^{7.45}$ is different from most class A GPCRs which mostly harbor an Asn at

the same position, but is conserved amongst chemokine receptors, and the binding of HMA in CCR2's sodium ion site indicates that amiloride binding allows for a certain variation in the amino acids that form the sodium ion site.

Dopamine receptors

The general trend amongst the dopamine receptor subtypes is an increase of the dissociation rate of orthosteric ligands by amiloride and its analogues, as found in a comprehensive study of the effect of amiloride, benzamil, and MIBA.[35] MIBA had the largest effect on the dissociation rates of the antagonists [^{3}H]SCH-23,390 at the human D_1 receptor and [^{3}H]spiperone at the human $D_{2(short)}$, $D_{2(long)}$, D_3, and D_4 dopamine receptors. As with other GPCRs the analogues with lipophilic moieties at the 5'-position were more potent than amiloride itself. At the D_1, $D_{2(short)}$, $D_{2(long)}$, and D_3 dopamine receptors the amilorides displaced the orthosteric antagonist [^{3}H]spiperone in both noncompetitive and competitive manners. This may indicate affinity of the amilorides to both the orthosteric and allosteric sites, and moreover a positive homotropic cooperativity was suggested based on a high Hill coefficient, i.e. amilorides binding at the allosteric site modulate the binding of amilorides to the orthosteric site positively.

The results at the D_2 receptor complemented results from other studies, in which similar dissociation rate increasing effects and mixed competitive/noncompetitive behavior were found. Amiloride competed with and increased the dissociation rate of antagonists [^{3}H]spiperone and [^{125}I]epidepride binding.[36] Amiloride, DMA, benzamil, EIA, MIBA, and HMA did so as well to the antagonist [^{3}H]spiperone at both the rat[37] and human[38] D_2 dopamine receptors, and of these amilorides HMA was the most potent amiloride (Figure 7). Agonists are modulated similarly as antagonists by amilorides at the rat D_2 and D_3 dopamine receptors, since amiloride, DMA, and MIBA decreased the potency of the agonist dopamine in inducing receptor activation in functional assays.[35, 39] At the D_4 receptor the allosteric effect of amiloride and its analogues was too small to be measured accurately, but an increase in antagonist [^{3}H]spiperone dissociation rate was still detected. As amilorides still inhibited binding of the orthosteric ligand the displacement was more competitive in nature.[35]

The amino acids forming the sodium ion site in the dopamine receptors are conserved compared to the adenosine and adrenergic receptors. Computational and mutagenesis

studies have confirmed the importance of Asp80$^{2.50}$, Ser121$^{3.39}$, Asn419$^{7.45}$, and Asn423$^{7.49}$ for the allosteric effects by sodium ions.[40-42] At the D_4 dopamine receptor mutation of Asp80$^{2.50}$ into Asn decreased MIBA affinity,[43] indicating that amilorides bind in the sodium ion site as well. It may be assumed that amilorides also bind in the sodium ion site of the other dopamine receptors, but this has not been confirmed yet.

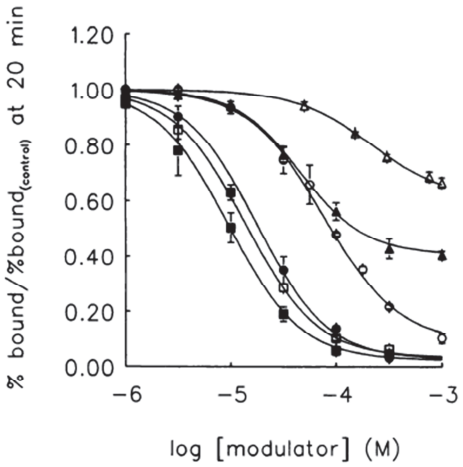

Figure 7. Concentration dependent dissociation modulation by amiloride and its analogues of [^{3}H]spiperone at the dopamine D_2 receptor after 20 minutes. (Δ-amiloride, ▲-benzamil, ○-DMA, ●-EIA, □-MIBA, ■-HMA). Amiloride modulates dissociation the least, while HMA and MIBA are the most effective modulators of dissociation. Reproduced with permission from Hoare and Strange (1996).[38]

Gonadotropin-releasing hormone receptor

The gonadotropin-releasing hormone (GnRH) receptor, also known as luteinizing hormone-releasing hormone receptor, is targeted by various drugs on the market for treatment of sex-hormone-dependent diseases such as breast or prostate cancer.[44, 45] These drugs are mostly peptidic agonists and antagonists that need to be administered by subcutaneous or intramuscular injections. The development of small molecule ligands that may replace these peptidic ligands is therefore desirable.[46] Earlier results had indicated allosteric modulation of GnRH stimulated luteinizing hormone release by sodium ions and amilorides.[47] In that light, the allosteric effects of amilorides on the GnRH receptor were investigated by Heitman et al.[48] Amiloride, benzamil, MCGMA, and phenamil had a

negligible effect on displacement of the peptide agonist [^{125}I]triptorelin at the GnRH receptor. However, DCB, MIBA, and HMA increased the dissociation rate of [^{125}I]triptorelin, with HMA having the strongest effect. In a luciferase assay, HMA acted as a purely insurmountable noncompetitive allosteric modulator as it only decreased the efficacy (E_{max}) of GnRH receptor activation by triptorelin and the endogenous ligand GnRH. Furthermore, it was demonstrated that the GnRH receptor harbors a second allosteric site next to the sodium ion site, since HMA did not compete with FD-1, an allosteric modulator with a distinct chemical structure, for the same allosteric site.

Muscarinic receptors

Amiloride effects have been found on muscarinic receptors in rat tissue preparations. Benzamil and HMA inhibited [^{3}H]pirenzepine binding at the muscarinic M_1 and [^{3}H]*N*-methylscopolamine binding at the muscarinic M_2 and M_3 receptors.[16] In rat trachea amiloride inhibited muscarinic M_3 receptor mediated smooth muscle contraction[49] by the endogenous agonist acetylcholine, by an insurmountable noncompetitive interaction as its efficacy (E_{max}) was reduced.[50] In rat parotic acini, which express the muscarinic M_3 receptor,[51] amiloride inhibited binding of the muscarinic receptor antagonist [^{3}H]N-methylscopolamine in a competitive manner.[52] In the recent relatively low resolution crystal structures of the muscarinic M_2 and M_3 receptors sodium ion binding was not detected,[53-55] but the amino acids making up the sodium ion site are perfectly conserved compared to adenosine and adrenergic receptors,[7] making amiloride binding to this site likely.

Serotonin receptors

Amiloride and analogues have been found to inhibit orthosteric ligand binding to serotonin receptors. Benzamil inhibited agonist [^{3}H]8-OH-DPAT binding at the rat 5-HT_{1A} receptor.[16] Amiloride and EIA inhibited agonist [^{3}H]5-carboxamidotryptamine binding at the human 5-HT_{1B} receptor.[56] In functional assays at the same receptor, amiloride inhibited receptor activation by agonist sumatriptan in a competitive manner, while EIA had intrinsic antagonistic activity as it inhibited forskolin-stimulated cAMP formation, albeit with a 15-fold higher EC_{50} value (200 μM) compared to its K_i in inhibiting [^{3}H]5-carboxamidotryptamine binding (13 μM).[56] Endogenous agonist [^{3}H]serotonin binding was inhibited by HMA at the rat 5-HT_{1C} receptor and by benzamil and HMA at the rat 5-HT_2

receptor.[16] Crystal structures of the agonist bound 5-HT_{1B} receptor[57] and the 5-HT_{2B} receptor,[58] again at relatively low resolution, did not reveal a bound sodium ion, but the well conserved amino acids of the sodium ion site compared to the other class A GPCRs[7] makes binding of amiloride in the same location likely.

Concluding remarks

This review summarizes the current knowledge of the allosteric effects of amiloride and its analogues on GPCRs. Allosteric effects of amilorides have been found on adenosine receptors, α-adrenergic receptors, the CCR2 chemokine receptor, dopaminergic receptors, the gonadotropin-releasing hormone receptor, the histamine H_1 receptor, muscarinic receptors, opioid receptors, and serotonin receptors.

Amiloride and its analogues seem to follow a few general 'rules' in their activity on these receptors. The propensity of amilorides to bind to the well conserved sodium ion site amongst GPCRs may explain these common behaviors. For most receptors, amiloride analogues with bulky lipophilic moieties on the 5'-position have greater affinity and potency than the unsubstituted parent compound. This has not been explained fully, but it is clear that in most GPCRs there is a hydrophobic pocket above the sodium ion site that can accommodate these lipophilic moieties. Most receptors allow substitution on the guanidinium group as well, with a good affinity in displacing orthosteric ligands, but with less or no effect on the dissociation of orthosteric ligands.

Another general 'rule' is the importance of $Asp^{2.50}$ for amiloride binding, just as for sodium ions. In the docking studies performed, the binding mode of amiloride and HMA was predicted in the sodium ion site of the adenosine A_{2A} receptor crystal structure and a CCR2 chemokine receptor homology model. The positively charged guanidinium group has a strong salt bridge interaction with $Asp^{2.50}$, underlining the great importance of this residue for amiloride binding as found before in mutagenesis studies. $Trp^{6.48}$ interacts with amilorides as well, in some cases hampering and in other cases accommodating amiloride binding. These interactions of amilorides with the amino acids of the sodium ion site are of interest since these have been shown to be important in receptor functionality, with $Asp^{2.50}$ and $Trp^{6.48}$ as most noticeable examples. Mutation of $Asp^{2.50}$ silences receptor activation in many GPCRs.[59] $Trp^{6.48}$ is noteworthy as a 'toggle switch' between the active

and inactive states of GPCRs,[60, 61] and in docking studies of the adenosine A_{2A} receptor amiloride and HMA seem to toggle it into its inactive rotamer (Chapter 3).

In contrast with these general 'rules', differences in the affinities, potencies, and modulatory behaviors of amilorides are quite diverse, even between receptors where the sodium ion site constitutes of exactly the same amino acids (i.e. adenosine, adrenergic, dopamine, and muscarinic receptors). To appreciate these differences it is important to discern between the different properties by which the allosteric effect of amilorides on orthosteric ligand binding may be described. In Table 1 we collected values for the different amilorides, of their affinity in displacing orthosteric ligands (IC_{50} or K_i), their effect on dissociation of orthosteric ligand ($k_{off}/k_{off(control)}$), and their potency for these dissociation effects (EC_{50}). This information also helps to understand whether the interaction of a particular amiloride with an orthosteric ligand is competitive or noncompetitive. If an amiloride inhibits orthosteric ligand binding, but does not affect its dissociation rate, the binding is mutually exclusive and the interaction is defined as competitive. If the dissociation rate is changed though, both the orthosteric ligand and amiloride can bind to the receptor at the same time and the interaction is deemed noncompetitive. Another way to confirm a noncompetitive interaction is by showing insurmountability of the inhibiting effect in radioligand saturation (B_{max} decrease) or functional assays (E_{max} decrease), as discussed for the chemokine CCR2, muscarinic M_3, and gonadotropin-releasing hormone receptor. However, these assays have been conducted far less than dissociation assays in amiloride research so we did not include them in Table 1.

In some cases, amilorides behave only as purely competitive inhibitors, while in other cases they behave as noncompetitive negative modulators, and a mixed behavior has also been observed. For some receptors the cause for mixed competitive/noncompetitive behavior was explained by a tendency of amilorides to bind both orthosteric and allosteric sites, but also in these cases the observed effect may be caused by binding in the sodium ion site only, where the competitive 'fraction' of the allosteric effect is caused by either an overlap of binding with the orthosteric site or a conformational change of the receptor by amiloride binding. The latter option is quite likely from the structural evidence provided by the recently elucidated crystal structures.

At some of the discussed receptors the modulatory effect by amilorides is probe-dependent, which has been described in other cases of allosteric modulation as well.[62, 63] Amilorides act as positive allosteric modulators for agonist binding and as negative modulators for antagonists at the α_{2A}-adrenergic and adenosine A_3 receptors. At the α_{2B}-adrenergic receptor different amilorides even exhibit both positive and negative modulatory effects. Some of the differences in affinity and modulatory effect may be caused by differences in the sodium ion site itself, but the substantial conservation of the sodium ion site residues amongst GPCRs makes it more likely that these differences are caused by variations in structural conformations elsewhere in the receptor.

Clinical application of amilorides targeting GPCRs is unlikely, due to their micromolar range affinities and lack of selectivity. However, it may be feasible to synthesize amiloride analogues with variations on the 5'-position to improve their affinity and selectivity for GPCRs. The possibility to use amilorides as tools to understand GPCR pharmacology is of greater interest though. For some of the amiloride-modulated receptors it has been confirmed that amilorides bind in the allosteric sodium ion site, and for the other receptors this is very likely. Indeed, allosteric modulation by sodium ions and amilorides often go together, and the investigation of confirmed sodium ion-modulated GPCRs that have not yet been researched for amiloride effects may be worthwhile. The sodium ion pocket region is important in the overall receptor activation of GPCRs, since mutation of sodium ion site residues has been shown to affect receptor functionality. With the ongoing expansion of the crystal structure pool of GPCRs, further study and knowledge of the mechanisms of amiloride modulation will help in understanding and appreciating the allosteric mechanism in GPCR functioning.

Table 1. Modulation of G protein-coupled receptors by amiloride and amiloride analogues. The given values reflect i) inhibitory potency or affinity for ligand displacement in radioligand binding assays or inhibition of ligand-induced receptor activation in functional assays, ii) modulatory potency of their effect on radioligand dissociation, and iii) fold change of dissociation rates of orthosteric ligands. References are in numbered superscript.

Receptor	**Orthosteric ligand** antagonist; *agonist*	**Amiloride (analogue)**	**Inhibitory potency or affinity**[a] - Displacement of orthosteric ligand by amiloride (analogue) in IC_{50} or K_i ± S.E.M. *(μM)*	**Modulatory potency** - Concentration-effect on dissociation of orthosteric ligand by amiloride (analogue) in EC_{50} ± S.E.M. *(μM)*	**Effect on dissociation** of orthosteric ligand by 100 μM[c] amiloride (analogue) in k_{off}/ $k_{off(control)}$ >1, Increase *<1, Decrease*
Adenosine A_1	[^{3}H]DPCPX	Amiloride	2.0 ± 0.2[B19]	-	1.5[Rd21]
			197 ± 23[R21]		
		Benzamil	0.65 ± 0.04[B19]	-	-
		CBDMB	1.2 ± 0.1[B19]	-	-
		DCB	1.6 ± 0.1[B19]	-	-
		DMA	8 ± 2[R21]	-	1.9[R21]
		HMA	0.41 ± 0.03[B19]	-	1.7[R21]
			22 ± 4[R21]		
		MBA	0.070 ± 0.004[B19]	-	-
		MGCMA	22 ± 1[B19]	-	-
		MIBA	0.16 ± 0.01[B19]	-	-
			13 ± 1[R21]		
		Phenamil	1.5 ± 0.1[B19]	-	-
	[^{3}H]PIA	Amiloride	2.4 ± 0.1[B19]	-	No effect[Rd21]
		Benzamil	0.85 ± 0.03[B19]	-	-
		CBDMB	4.0 ± 0.4[B19]	-	-
		DCB	2.7 ± 0.2[B19]	-	-
		DMA	-	-	No effect[R21]
		HMA	0.50 ± 0.03[B19]	-	No effect[R21]
		MBA	0.09 ± 0.01[B19]	-	-
		MGCMA	16 ± 1[B19]	-	-
		MIBA	0.20 ± 0.01[B19]	-	-
		Phenamil	2.3 ± 0.1[B19]	-	-

Adenosine A_{2A}	[^{3}H]ZM-241,385	Amiloride	9.7 ± 1.1^{R20}	-	1.2^{Rd20}
		Benzamil	2.2 ± 0.3^{R20}	-	2.4^{Rd20}
		HMA	3.3 ± 0.5^{R20}	-	12^{Rd20}
		MGCMA	89 ± 13^{R20}	-	1.2^{Rd20}
		MIBA	3.0 ± 0.2^{R20}	-	5.7^{Rd20}
		Phenamil	2.6 ± 0.4^{R20}	-	1.9^{Rd20}
	[^{3}H]CGS-21,680	Amiloride	-	-	No effect^{Rd21}
		DMA	-	-	No effect^{R21}
		HMA	-	-	No effect^{R21}
Adenosine A_3	[^{3}H]PSB-11	Amiloride	82 ± 7^{H21}	-	No effect^{Hd21}
		DMA	13 ± 2^{H21}	-	1.3^{H21}
		HMA	6 ± 1^{H21}	-	2.3^{H21}
		MIBA	8 ± 1^{H21}	-	1.6^{H21}
	[^{125}I]I-AB-MECA	Amiloride	>100^{R21}	-	No effect^{Hd21}
		DMA	20 ± 3^{R21}	-	*0.80*^{H21}
		HMA	7 ± 1^{R21}	-	*0.53*^{H21}
		MIBA	7 ± 2^{R21}	-	*0.59*^{H21}
α_{1A}-Adrenergic	[^{3}H]Prazosin	Amiloride	11 ± 2^{H24}	-	1.2^{H24}
		Benzamil	0.8 ± 0.1^{H24}	-	1.7^{H24}
		DMA	0.82 ± 0.03^{H24}	-	1.5^{H24}
		EIA	2.7 ± 0.3^{H24}	-	2.2^{H24}
		HMA	1.1 ± 0.2^{H24}	-	5.5^{H24}
		MIBA	0.49 ± 0.07^{H24}	-	2.4^{H24}
α_{2A}-Adrenergic	[^{3}H]Yohimbine	Amiloride	30 ± 2^{H29}	-	2.0^{He29}
		A-EIA-AS	-	40^{P28}	> 1^{P28}
		Benzamil	3.5 ± 0.7^{H29}	-	No effect^{He29}
		DMA	3.6 ± 0.1^{H29}	-	5.3^{He29}
	[^{3}H]Rauwolscine		-	-	6.3^{He29}
	[^{3}H]RX-821,002	DMA	-	-	7.1^{He29}
	[^{3}H]Yohimbine	EIA	1.7 ± 0.2^{H29}	50^{P28}	> 1^{P28}
					155^{He29}
		HMA	0.21 ± 0.00^{H29}	-	138^{He29}
	[^{3}H]Rauwolscine		-	-	57^{He29}
	[^{3}H]Yohimbine	MIBA	0.56 ± 0.01^{H29}	-	101^{He29}
	[^{3}H]UK-14,304	Amiloride	25 ± 0.2^{H30}	-	*0.67*^{He30}
		DMA	3.2 ± 0.2^{H30}	-	*0.77*^{He30}
		HMA	0.18 ± 0.02^{H30}	-	*0.37*^{He30}

α_{2B}-Adrenergic	[^{3}H]Rauwolscine	CBDMB	-	-	< 1^{R31}
		EIA	-	-	> 1^{R31}
		MIBA	-	-	> 1^{R31}
β_1-Adrenergic	[^{125}I]Iodocyano-pindolol	Amiloride	83 ± 14^{R23}	-	-
β_2-Adrenergic	[^{125}I]Iodocyano-pindolol	Amiloride	60^{R23}	-	-
CCR2	[^{3}H]INCB3344	Amiloride	No effect^{Hf32}	-	-
		Benzamil	No effect^{Hf32}	-	-
		HMA	79^{H32}	-	1.25^{H32}
		MCGMA	No effect^{Hf32}	-	-
		MIBA	158^{H32}	-	-
		Phenamil	No effect^{Hf32}	-	-
	[^{3}H]CCR2-RA-[R]^{g}	Amiloride	No effect^{Hf32}	-	-
		Benzamil	No effect^{Hf32}	-	-
		HMA	79^{H32}	-	1.36^{H32}
		MCGMA	No effect^{Hf32}	-	-
		MIBA	126^{H32}	-	-
		Phenamil	No effect^{Hf32}	-	-
	[^{125}I]CCL2	HMA	-	-	9.7^{H32}
Dopamine D_1	[^{3}H]SCH-23,390	Amiloride	49 ± 1^{H35}	>1000^{H35}	-
		Benzamil	1.6 ± 0.5^{H35}	74 ± 8^{H35}	-
		MIBA	4.4 ± 0.2^{H35}	13 ± 1^{H35}	26^{He35}
Dopamine D_2	[^{125}I]Epidepride	Amiloride	-	-	2.5^{Ri36}
	[^{3}H]Spiperone		390 ± 4^{H35}	215 ± 35^{R38}	1.5^{Ri36}
			-	100 ± 10^{H35}	2.7^{Rj38}
		Benzamil	25 ± 2^{H35}	46 ± 4^{R38}	4.8^{Rk38}
				29 ± 7^{H35}	
		DMA	-	76 ± 8^{R38}	8.4^{Rk38}
		EIA	-	20 ± 5^{R38}	18^{Rl38}
		HMA	-	10 ± 2^{R38}	16^{Rl38}
		MIBA	6.6 ± 0.4^{H35}	14 ± 1^{R38}	14^{Rl38}
				2.1 ± 0.2^{H35}	88^{He35}
	Dopamine	Amiloride	29^{Rm39}	-	-
		DMA	1.4^{Rm39}	-	-
		MIBA	0.9^{Rm39}	-	-
			0.6 ± 0.2^{Hn35}		

Dopamine D_3	[^{3}H]Spiperone	Amiloride	120 ± 7^{H35}	43 ± 3^{H35}	-
		Benzamil	16 ± 1^{H35}	15 ± 2^{H35}	-
		MIBA	1.7 ± 0.1^{H35}	0.29 ± 0.14^{H35}	18^{He35}
	Dopamine	MIBA	1.8^{Ro39}	-	-
Dopamine D_4	[^{3}H]Spiperone	Amiloride	280 ± 30^{H35}	420 ± 4^{H35}	-
		Benzamil	6.1 ± 0.4^{H35}	28 ± 2^{H35}	-
		MIBA	1.3 ± 0.2^{H35}	22 ± 5^{H35}	$> 1^{He35}$
GnRH	*[^{125}I]Triptorelin*	Amiloride	$> 100^{H48}$	-	-
		Benzamil	$> 100^{H48}$	-	-
		DCB	30 ± 3^{H48}	-	1.7^{H48}
		MIBA	39 ± 7^{H48}	-	2.1^{H48}
		HMA	29 ± 3^{H48}	49 ± 7^{H48}	2.5^{H48}
		MCGMA	$> 100^{H48}$	-	-
		Phenamil	$> 100^{H48}$	-	-
Histamine H_1	[^{3}H]Mepyramine	Amiloride	$> 10^{R16}$	-	-
		Benzamil	3.2 ± 0.2^{R16}	-	-
		HMA	5.6 ± 1.2^{R16}	-	-
Muscarinic M_1	[^{3}H]Pirenzepine	Amiloride	$>10^{R16}$	-	-
		Benzamil	2.9 ± 0.7^{R16}	-	-
		HMA	3.6 ± 1.0^{R16}	-	-
Muscarinic M_2	[^{3}H]*N*-methyl-scopolamine	Amiloride	$> 10^{R16}$	-	-
		Benzamil	5.8 ± 1.1^{R16}	-	-
		HMA	2.9 ± 0.5^{R16}	-	-
Muscarinic M_3	[^{3}H]*N*-methyl-scopolamine	Amiloride	50^{R52}	-	-
		Benzamil	2.8 ± 0.5^{R16}	-	-
		HMA	4.7 ± 0.8^{R16}	-	-
	Acetylcholine	Amiloride	478^{Rq50}	-	-
δ-Opioid	*[^{3}H]DADLE*	Amiloride	$> 10^{R16}$	-	-
		Benzamil	$> 10^{R16}$	-	-
		HMA	1.0 ± 0.2^{R16}	-	-
κ-Opioid	*[^{3}H]Ethyl-ketazocine*	Amiloride	$> 10^{R16}$	-	-
		Benzamil	$> 10^{R16}$	-	-
		HMA	3.9 ± 0.6^{R16}	-	-
μ-Opioid	[^{3}H]Naloxone	Amiloride	$> 10^{R16}$	-	-
		Benzamil	1.1 ± 0.4^{R16}	-	-
		HMA	0.06 ± 0.02^{R16}	-	-

Serotonin 5-HT_{1A}	*[^{3}H]8-OH-DPAT*	Amiloride	$> 10^{R16}$	-	-
		Benzamil	1.9 ± 0.3^{R16}	-	-
		HMA	$> 10^{R16}$	-	-
Serotonin 5-HT_{1B}	*[^{3}H]5-Carboxa-midotryptamine*	Amiloride	20^{H56}	-	-
	Sumatriptan		35^{Hs56}	-	-
	[^{3}H]Serotonin	Benzamil	$> 10^{R16}$	-	-
	[^{3}H]5-Carboxa-midotryptamine	EIA	13^{H56}	-	-
	[^{3}H]Serotonin	HMA	$>10^{R16}$	-	-
Serotonin 5-HT_{1C}	*[^{3}H]Serotonin*	Amiloride	$>10^{R16}$	-	-
		Benzamil	$>10^{R16}$	-	-
		HMA	6.7 ± 1.2^{R16}	-	-
Serotonin 5-HT_{1D}	*[^{3}H]Serotonin*	Amiloride	$> 10^{R16}$	-	-
		Benzamil	$> 10^{R16}$	-	-
		HMA	$> 10^{R16}$	-	-
Serotonin 5-HT_2	*[^{3}H]Serotonin*	Amiloride	$> 10^{R16}$	-	-
		Benzamil	1.4 ± 0.1^{R16}	-	-
		HMA	0.40 ± 0.06^{R16}	-	-

-: Not determined.

a: IC_{50} values determined with concentrations of orthosteric radioligands around their K_D.

B: Bovine receptor.

c: In presence of 100 µM amiloride (analogue) except when stated otherwise.

d: In presence of 1 mM amiloride (analogue).

e: Calculated for the amiloride (analogue) occupied receptor.

f: No displacement of orthosteric ligand by 100 µM amiloride (analogue).

g: [^{3}H]CCR2-RA-[R] is an 'intracellular antagonist' as it binds to the second intracellular site of the chemokine CCR2 receptor.

H: Human receptor.

i: In presence of 500 µM amiloride (analogue).

j: In presence of 3.16 mM amiloride (analogue).

k: In presence of 1 mM amiloride (analogue).

l: In presence of 316 µM amiloride (analogue).

m: Inhibition by amiloride (analogue) of dopamine-stimulated increase in extracellular acidification rate in cells expressing the dopamine D_2 receptor.

n: Inhibition by MIBA of dopamine-stimulated [^{35}S]GTPγS binding to dopamine D_2 receptors.

o: Inhibition by MIBA of dopamine-stimulated increase in extracellular acidification rate in cells expressing the dopamine D_3 receptor.

P: Porcine receptor.

q: Modulation by amiloride of acetylcholine-induced contractions of rat tracheal smooth muscle, which expresses the muscarinic M_3 receptor.

R: Rat receptor.

s: Inhibition by amiloride of the sumatriptan-induced reduction of cAMP formation stimulated by forskolin in cells expressing the Serotonin 5-HT_{1B} receptor.

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