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 5

5’-Substituted amiloride derivatives as allosteric modulators binding in the sodium ion pocket of the adenosine A_{2A} receptor

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Abstract

The sodium ion site is an allosteric site conserved among many G protein-coupled receptors (GPCRs). Amiloride **1** and 5-(*N,N*-hexamethylene)amiloride **2** (HMA) supposedly bind in this sodium ion site and can influence orthosteric ligand binding. The availability of a high resolution X-ray crystal structure of the human adenosine A_{2A} receptor ($hA_{2A}AR$), in which the allosteric sodium ion site was elucidated, makes it an appropriate model receptor for investigating the allosteric site. In this study, we report the synthesis and evaluation of novel 5'-substituted amiloride derivatives as $hA_{2A}AR$ allosteric antagonists. The potency of the amiloride derivatives was assessed by their ability to displace orthosteric radioligand [^{3}H]4-(2-((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*]-[1,3,5]triazin-5-yl)amino)ethyl)phenol ([^{3}H]ZM-241,385) from both the wild-type and sodium ion site W246A mutant $hA_{2A}AR$. 4-Ethoxyphenethyl-substituted amiloride **12l** was found to be more potent than both amiloride and HMA, and the shift in potency between the wild-type and mutated receptor confirmed its likely binding to the sodium ion site.

Introduction

G protein-coupled receptors (GPCRs) are proteins that consist of seven transmembrane helices and reside in cell membranes. They are able to transfer a multitude of signals, conducted by carriers such as light, hormones, and small molecules, from the extracellular environment to the interior of the cell. The superfamily is predicted to contain approximately 800 different receptors, of which a considerable number are yet to be characterized.[1] Approximately 30% to 40% of drugs currently on the market target GPCRs, underlining their importance in physiology and medicine.[2] Most of these drugs are orthosteric ligands that target endogenous ligand-binding sites of GPCRs. However, GPCRs also contain allosteric binding sites, and ligands that bind to these can influence endogenous GPCR signaling. The use of allosteric ligands as drugs has several potential advantages, such as a more specific drug effect in time and location, due to their ability to modulate orthosteric ligand responses.[3, 4]

The sodium ion binding site is an allosteric site that is well-conserved among GPCRs. The first X-ray crystal structure of a GPCR containing an allosterically bound sodium ion was of the human adenosine A_{2A} receptor ($hA_{2A}AR$),[5] which was followed by several other GPCR structures with a sodium ion.[6-9] At physiological concentrations of sodium ions, approximately 80% of $hA_{2A}ARs$ are occupied by the cation, which reduces orthosteric agonist binding (Chapter 3). Mutation of amino acids that make up the sodium ion site changes the activation characteristics of the $hA_{2A}AR$, highlighting the importance of the site for receptor function (Chapter 4).

Amiloride **1** and its derivative 5-(*N,N*-hexamethylene)amiloride **2** (HMA) are known to bind to the sodium ion site of several GPCRs,[10-13] including adenosine receptors.[14, 15] Amiloride and HMA act on other targets as well, such as the enzyme urokinase-type plasminogen activator (uPA).[16, 17] Furthermore, amiloride is used clinically as a potassium-sparing diuretic drug, where its action arises predominantly through blockade of renal epithelial sodium channels (ENaCs).[18] The positively charged guanidinium moiety of amiloride and HMA may explain their propensity to bind to negatively charged target sites and their pharmacological promiscuity more generally. Nevertheless, their affinity for the sodium ion site of GPCRs makes amiloride and its derivatives potentially useful pharmacological tools for probing molecular mechanisms of GPCR activation and regulation, as demonstrated in Chapter 4. The increased affinity of HMA compared to that

of amiloride for the sodium ion site of the $hA_{2A}AR$[14] suggests that there is potential to further elaborate the amiloride core at its 5' position, possibly yielding derivatives with higher potency.

In a study by Matthews et al., several new amiloride derivatives with substituents at the 5' position were found to be active against uPA.[16] In our current study, we tested a selection of these for their activity at the $hA_{2A}AR$ by measuring their ability to displace orthosteric radioligand [^{3}H]4-(2-((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*]-[1,3,5]triazin-5-yl)amino)ethyl)phenol ([^{3}H]ZM-241,385).[19] After identifying the most potent derivatives, we designed and synthesized novel 5'-substituted amiloride analogues and evaluated their $hA_{2A}AR$ activity. From these analogues, compounds were identified that were equipotent or more potent than reference compound HMA.

Compounds were also evaluated using an $hA_{2A}AR$ mutant, where the Trp246$^{6.48}$ 'toggle switch'[20] (numbering in superscript according to Ballesteros and Weinstein)[21] was replaced by Ala. This tryptophan forms part of the sodium ion pocket, and mutation to alanine has been shown to significantly increase the potency of amiloride and HMA (Chapter 4), making the W246A$^{6.48}$ mutant receptor a useful tool for assessing the binding of ligands in the sodium ion pocket. Finally, the most potent derivative was docked into the sodium ion site of the $hA_{2A}AR$ crystal structure (PDB: 4EIY).[5] Comparisons of the binding of compounds to the wild-type and W246A$^{6.48}$ mutant receptors and docking of the most potent derivatives into the $hA_{2A}AR$ structure provided insights into the molecular interactions occurring between the amiloride derivatives and the sodium ion site.

Materials and methods

Materials

[^{3}H]ZM-241,385 (50 Ci/mmol) was obtained from ARC Inc. (St. Louis, MO, USA). ZM-241,385[22] was obtained from Ascent Scientific (Bristol, UK). NECA, amiloride, HMA, and bovine serum albumin (BSA) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Adenosine deaminase (ADA) was purchased from Boehringer Mannheim (Mannheim, Germany). Bicinchoninic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL, USA). HEK293 cells stably expressing the $hA_{2A}AR$ (HEK293-$hA_{2A}AR$) were a gift from Dr. J. Wang (Biogen/IDEC,

Cambridge, MA, USA). All other chemicals were of analytical grade and obtained from standard commercial sources.

Chemistry

2-(4-(Allyloxy)phenyl)ethan-1-amine Hydrochloride **(20).** To a suspension of $LiAlH_4$ (41 mmol) in dry THF (20 mL) at 0 °C was added nitro-styrene **19** (2.54 g, 12.4 mmol) slowly. The mixture was stirred at 0 °C for 1 h and then heated at reflux for 2 h. The reaction was cooled to 0 °C and water (6 mL) was added. The mixture was diluted with THF (10 mL) and filtered. The filtrate was extracted with EtOAc/H_2O, and the organic layer was washed with water and dried using $MgSO_4$. The volatiles were removed in vacuo to afford the free amine; HCl (1 M in EtOAc, 5 mL) was added and then evaporated, yielding **20** (1.65 g, 78%) as its HCl salt. ^{1}H NMR (400 MHz, CD_3OD) 7.19 (d, *J* = 8.3 Hz, 2H), 6.92 (d, *J* = 8.3 Hz, 2H), 6.11–5.99 (m, 1H), 5.39 (δ, *J* = 17.3 Hz, 1H), 5.24 (d, *J* = 10.6 Hz, 1H), 4.53 (d, *J* = 4.7 Hz, 2H), 3.13 (t, *J* = 7.6 Hz, 2H), 2.89 (t, *J* = 7.6 Hz, 2H).

General Procedure for the Synthesis of Compounds **12a-12p.** To a solution of **21** (0.3 mmol) and corresponding amine or amine hydrochloride (0.3 mmol) in dry *N,N*-dimethylformamide (DMF) (5 mL) was added DiPEA (0.75 mmol in the case of the free amine or 1.05 mmol in the case of the amine hydrochloride) at room temperature. The resulting reaction mixture was stirred at 100 °C for 1-2 h. Upon reaction completion (TLC), the reaction mixture was cooled to room temperature, poured into ice-water and extracted with AcOEt/H_2O. The organic layer was dried over $MgSO_4$ and, after concentration in vacuum, yielded the desired free base of the final compound with a purity of at least 95%. The free base was dissolved in 6 mL of 1 N HCl/AcOEt solution, stirred at room temperature for 2 h, and evaporated to dryness to give the hydrochloride salt of the desired product as a powder (compounds **12a-f**, **h-k**, **n**, and **p**).

The other compounds were further purified by preparative HPLC (using the free base), which was performed on a Shimadzu HPLC–ultraviolet (UV) system with a diode array detector using a Gemini 5 μm C18 110A column (100 × 10 mm, 5 μm) and a linear gradient from 1 to 99% mobile phase B. Mobile phase A consisted of H_2O, and mobile phase B consisted of CH_3CN/1% TFA in H_2O. The flow rate was 5 mL/min. The TFA salt of the desired product was obtained as a yellow powder (compounds **12g**, **l-m**, and **o**).

3-Amino-*N*-carbamimidoyl-6-chloro-5-((2,3-dihydro-1*H*-inden-2-yl)amino)pyrazine-2-carboxamide Hydrochloride **(12a).** Yield = 67%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.64 (s, 1H), 8.61 (br s, 2H), 8.44 (br s, 2H), 8.02 (d, *J* = 7.2 Hz, 1H), 7.58 (br s, 2H), 7.23–7.14 (m, 4H), 4.87–4.77 (X part of ABX system, sextet, *J* = 8.0 Hz, 1H), 3.62 (A part of ABX system, dd, *J* = 16.0, 7.6 Hz, 2H), 3.06 (B part of ABX system, dd, *J* = 15.6, 8.0 Hz, 2H). ESI-MS *m/z:* 346.13 $[M+H]^+$; t_R = 7.38 min.

Biology

HEK293 cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS), 50 µg/ml streptomycin and 50 IU/ml penicillin at 37 °C and 7% CO_2. Cells were subcultured twice a week at a ratio of 1:20 on 10 cm ø plates. A point mutation corresponding to W246A$^{6.48}$ was introduced into the wild-type hA_{2A}AR-plasmid DNA (FLAG-tag at the N-terminus, in pcDNA3.1) by BaseClear (Leiden, The Netherlands). Cells were transfected with 1 µg per plate of the resulting plasmid using the calcium phosphate precipitation method.[23] HEK293 cells stably expressing the wild-type receptor were grown in the same medium but with the addition of G-418 (200 µg/ml).

Membranes were prepared as follows. HEK293 cells were detached from plates 48 h after transfection (cells transiently expressing hA_{2A}AR-W246A$^{6.48}$) or from confluent plates (cells stably expressing hA_{2A}AR-WT) by scraping them into 5 ml PBS, collected and centrifuged at 700 g (3000 rpm) for 5 min. Pellets derived from 20 plates were pooled and resuspended in 16 ml of ice-cold assay buffer (50 mM Tris-HCl, pH 7.4). An Ultra-Turrax was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100000 g (31000 rpm) in a Beckman Optima LE-80K ultracentrifuge at 4 °C for 20 min. The pellet was resuspended in 8 ml of Tris buffer, and the homogenization and centrifugation steps were repeated. Assay buffer (4 ml) was used to resuspend the pellet, and adenosine deaminase (ADA) was added (0.8 IU/ml) to break down endogenous adenosine. Membranes were stored in 250 µL aliquots at -80 °C. Membrane protein concentrations were measured using the BCA method.[24]

For [^{3}H]ZM-241,385 displacement binding experiments membranes with stably expressed hA_{2A}AR-WT (15 µg of total protein) or with transiently expressed hA_{2A}AR-W246A$^{6.48}$ (4 µg) were used. These protein amounts ensured that total binding to the membrane preparations was less than 10% of the total radioactivity added in order to

prevent radioligand depletion. Membrane aliquots were incubated in a total volume of 100 µl of assay buffer at 25 °C for 2 h. [^{3}H]ZM-241,385 was used at a concentration of 2.5 nM. Total binding was determined in the presence of [^{3}H]ZM-241,385 without addition of other compounds. Nonspecific binding was determined in the presence of 100 µM NECA (hA_{2A}AR-WT) or 10 µM ZM-241,385 (hA_{2A}AR-W246A$^{6.48}$) and represented less than 10% of the total binding. For single-point assays, radioligand displacement experiments were performed in the presence or absence of 10 µM amiloride **1**, HMA **2**, or compounds **3-18**. For concentration-effect curves, radioligand displacement experiments were performed in the absence or presence of increasing concentrations of HMA **2** or **12h**, **i**, **k**, or **l**. For radioligand dissociation experiments, membrane preparations were first allowed to reach binding equilibrium with [^{3}H]ZM-241,385 for 1 h at 4 °C. Dissociation was started by addition of 100 µM NECA with or without 100 µM HMA **2** or **12h**, **i**, **k**, or **l** (hA_{2A}AR-WT), or addition of 10 µM ZM-241,385 with or without 10 µM HMA **2** or **12h**, **i**, **k**, or **l** (hA_{2A}AR-W246A$^{6.48}$) at each time point. The time points were distributed over 180 min (hA_{2A}AR-WT) or 30 min (hA_{2A}AR-W246A$^{6.48}$). Incubations were terminated by rapid vacuum filtration to separate the bound radioligand from free radioligand through 96-well GF/B filter plates using a Filtermate-harvester (PerkinElmer Life Sciences). Filters were subsequently washed three times with ice-cold assay buffer. The filter-bound radioactivity was determined by scintillation spectrometry using a PE 1450 Microbeta Wallac Trilux scintillation counter (PerkinElmer Life Sciences).

The radioligand binding data was analyzed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The displacement curves were fitted to a one-state site binding model, and the dissociation curves were fitted to a one-phase dissociation model. A one-way ANOVA with Dunnett's post hoc test was performed to test for significant differences.

Computational receptor modeling

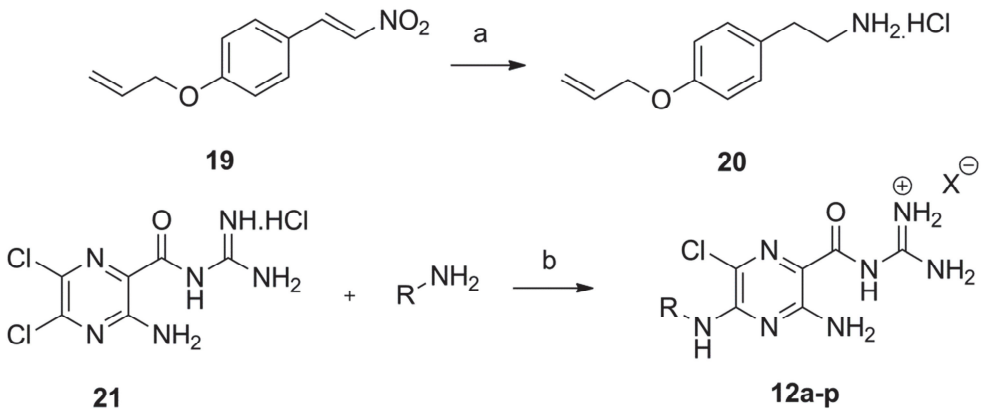
All structure-based studies were performed in the Schrödinger suite.[25] The inactive structure of the A_{2A}AR in complex with ZM-241,385 and a sodium ion (PDB: 4EIY)[5] was used as the basis for docking. Docking of **12h**, **i**, **k**, and **l** was based on the docking pose of HMA described in Chapter 3. To allow for side chain rearrangements, we used core-constrained docking (with the guanidinium moiety as the core) with a decreased van der Waals (vdW) sphere (0.5 instead of 0.8). The vdW sphere was decreased to allow for an

induced fit in the sodium pocket. Subsequently, we optimized this pose using an exhaustive hierarchical optimization procedure available in Prime,[26] which has been used previously to model induced fit effects.[27] Figures were rendered using PyMol.[28]

Results

Chemistry

The synthesis of compounds **3–18** has been described previously.[16] New compounds **12a-p** were synthesized in a similar manner, through addition of the appropriate phenethylamine onto the amiloride core **21** in the presence of base (Scheme 1). The compounds were obtained as hydrochloride or trifluoroacetate salts depending on the purification method employed. Amine hydrochloride salt **20**, which has not been previously described, was synthesized by reducing nitrostyrene **19**[29] with $LiAlH_4$ followed by treatment with HCl.[30]



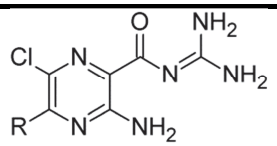
Scheme 1. Synthesis of phenethyl amiloride derivatives 12a-p. Reagents and conditions: a) i. $LiAlH_4$, THF, 0 °C to 76 °C, 2 h, ii. HCl, 78%; b) i. DiPEA, DMF, 100 °C, ii. HCl (X^- = Cl^-) or prep HPLC purification (X^- = $CF_3CO_2^-$), 1–75%.

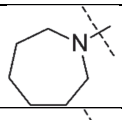
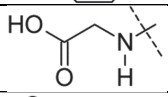
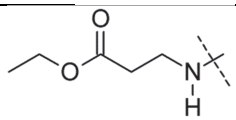
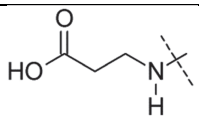
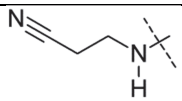
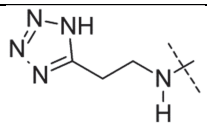
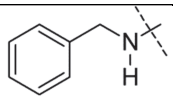
Biology and structure-activity relationships

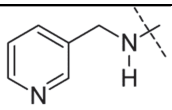
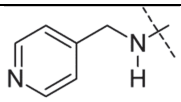
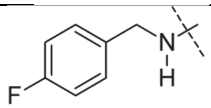
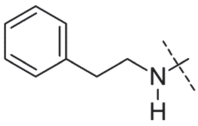
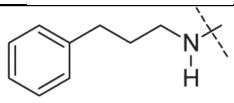
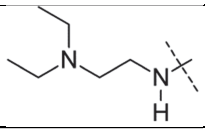
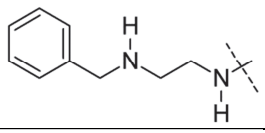
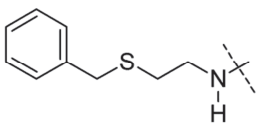
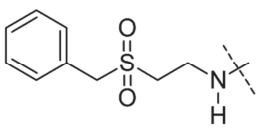
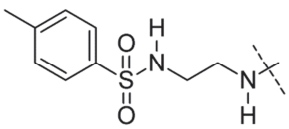
Single point displacement assays were performed in which [^{3}H]ZM-241,385 binding to the hA_{2A}AR was displaced by 10 µM of compounds **1–18** (Table 1) and **12a-p** (Table 2). Reference compounds amiloride **1** and HMA **2** displaced [^{3}H]ZM-241,385 binding from the wild-type receptor by 29 % and 70 %, respectively, and from the W246A$^{6.48}$ mutant receptor by 89 % and 98 %. Of amiloride derivatives **3–18,** compounds **3-5**, **7**, **8**, **10**, **11**, and **14–18** displaced less [^{3}H]ZM-241,385 than amiloride at both the wild-type and W246A$^{6.48}$ receptors. Carboxylate derivatives **3** and **5**, and tetrazole **7** showed no or marginal displacement of [^{3}H]ZM-241,385 from both receptors. Carboxylate ester **4** and diethylamino-ethyl derivative **14** showed negligible displacement of [^{3}H]ZM-241,385 binding from the wild-type receptor, but they displaced approximately one-half of [^{3}H]ZM-241,385 binding from the W246A$^{6.48}$ mutant. Nitrile derivative **6** displaced slightly more [^{3}H]ZM-241,385 binding from the wild-type receptor (35%) than amiloride, but it displaced less than amiloride at the W246A$^{6.48}$ receptor (83%). Benzylic derivatives **8-11** did not or marginally displaced [^{3}H]ZM-241,385 binding from the wild-type receptor, except for 3-pyridyl derivative **9**, which performed similarly to amiloride. However, compounds **8**, **9**, and **11** were able to displace [^{3}H]ZM-241,385 binding from the mutant receptor by approximately 80% (benzyl **8**) and 50% (3-pyridyl **9** and 4-fluorobenzyl **11**). 4-Pyridyl derivative **10** showed minor radioligand displacement at the mutated receptor.

Extension of the carbon chain to phenylethyl derivative **12** yielded the largest decrease in orthosteric [^{3}H]ZM-241,385 binding to both the wild-type (46%) and W246A$^{6.48}$ mutant (99%) receptors. Further extension of the carbon chain to phenylpropyl derivative **13** produced slightly lower reductions in [^{3}H]ZM-241,385 binding to the wild-type (40%) and mutant (91%) receptors relative to that with **12**. Increased linker sizes and inclusion of heteroatoms (compounds **15**-**18**) marginally displaced [^{3}H]ZM-241,385 binding from the wild-type receptor. However, these compounds were able to displace [^{3}H]ZM-241,385 binding from the W246A$^{6.48}$ mutant receptor by approximately 80% (sulfone **17**), 65% (sulfide **16** and sulfonamide **18**), and 35% (amine **15**).

Table 1. $hA_{2A}AR$ binding of amiloride derivatives. % [^{3}H]ZM-241,385 binding to the $hA_{2A}AR$-WT and -W246A$^{6.48}$ in the presence of 10 µM amiloride **1**, HMA **2**, or amiloride derivatives **3-18**. The concentration of [^{3}H]ZM-241,385 was 2.5 nM. 100 % [^{3}H]ZM-241,385 binding was determined in the absence of any amiloride, while 0 % binding was determined as nonspecific binding of [^{3}H]ZM-241,385 assessed in the presence of 100 µM NECA (WT) or 10 µM ZM-241,385 (W246A$^{6.48}$). Values are means ± standard error of mean (SEM) of at least three independent assays performed in duplicate.

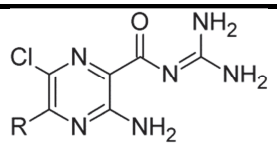


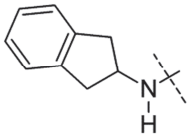
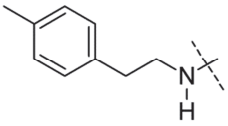
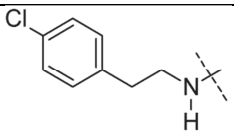
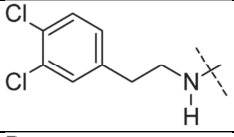
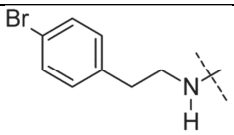
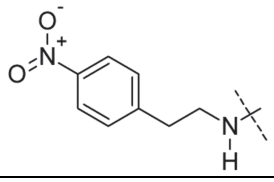
Compound	R	% [^{3}H]ZM-241,385 binding ± SEM to $hA_{2A}AR$ in the presence of 10 µM compound	
		WT	W246A$^{6.48}$
Amiloride **1**	NH_2	71 ± 2	11 ± 1
HMA **2**		30 ± 2	1.8 ± 0.3
3		88 ± 1	111 ± 9
4		87 ± 7	48 ± 10
5		96 ± 3	70 ± 9
6		65 ± 3	17 ± 3
7		99 ± 4	101 ± 2
8		77 ± 4	21 ± 4

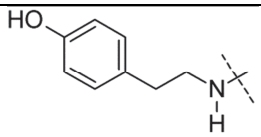
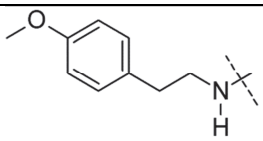
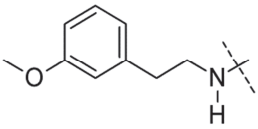
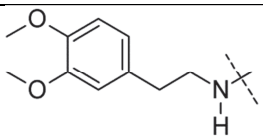
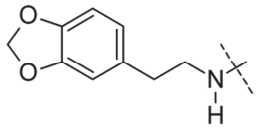
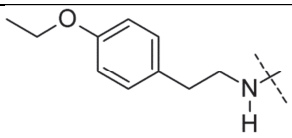
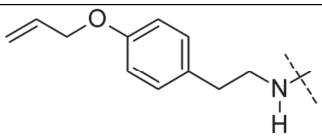
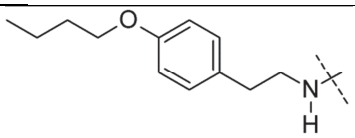
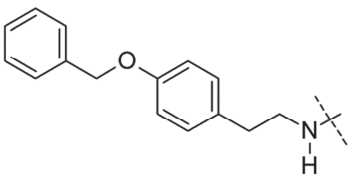
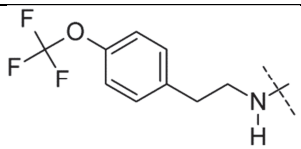
9		71 ± 3	46 ± 3
10		87 ± 4	73 ± 4
11		96 ± 6	48 ± 5
12		54 ± 3	1.1 ± 0.4
13		60 ± 2	9.0 ± 1.9
14		85 ± 3	53 ± 7
15		90 ± 3	65 ± 8
16		84 ± 3	35 ± 2
17		82 ± 4	18 ± 2
18		85 ± 3	32 ± 5

After observing increased potency with phenylethyl derivative **12**, we synthesized and tested compounds **12a**-**p** carrying different substituents on the phenyl moiety (Table 2). Compounds **12a**, **12c-g**, and **12n-p** displaced less [^{3}H]ZM-241,385 binding than **12** from both the wild-type and W246A$^{6.48}$ mutant hA_{2A}AR. These derivatives performed similarly to amiloride at the wild-type receptor, whereas they performed better at the mutated receptor, with close to 100% radioligand displacement, with the exception of 4-chlorophenethyl derivative **12c** and 3,4-dichlorophenethyl derivative **12d**, which caused minor displacement of [^{3}H]ZM-241,385 binding from the wild-type receptor (8% and 18%, respectively) and the lowest, although significant, displacement from the mutated receptor (68% and 89%, respectively) of the phenethyl derivatives. Compared to parent **12**, 2-(*p*-tolyl)ethyl derivative **12b** displaced similar amounts of radioligand from the wild-type and W246A$^{6.48}$ receptors. 4-Bromo **12e**, 4-nitro **12f**, and 4-hydroxy **12g** substituted phenethyl derivatives showed lower activity than **12** at both the wild-type and mutant receptors. Ether substituted phenethyl derivatives **12h**-**m** displaced more radioligand than **12** from the wild-type receptor, and reached close to 100% radioligand displacement at the W246A$^{6.48}$ receptor. 4-Methoxyphenethyl derivative **12h** displaced 69% of radioligand binding from the wild-type receptor, thus performing similarly to HMA, whereas changing the methoxy position to give 3-methoxyphenethyl derivative **12i** resulted in less radioligand displacement (59%). Addition of a second methoxy group, as in 3,4-dimethoxyphenethyl derivative **12j**, decreased radioligand displacement from the wild-type receptor even more (49%). Dioxolyl derivative **12k** increased radioligand displacement from the wild-type receptor (65%), bringing its effect close to that of HMA. Elongation of **12h** by a methyl group resulting in 4-ethoxyphenethyl derivative **12l** yielded the most effective amiloride derivative of this series, reducing [^{3}H]ZM-241,385 binding from the wild-type receptor by 73%. Further elongation of the ethoxy substituent to give 4-(allyloxy)phenethyl derivative **12m** decreased radioligand displacement again, whereas the even bulkier ether substituted phenethyl derivatives **12n**-**p** displaced less radioligand than **12**.

Table 2. $hA_{2A}AR$ binding of amiloride phenethyl substituted derivatives. % [^{3}H]ZM-241,385 binding to the $hA_{2A}AR$-WT and -W246A$^{6.48}$ in the presence of 10 µM amiloride derivatives **12a-p**. The concentration of [^{3}H]ZM-241,385 was 2.5 nM. 100 % [^{3}H]ZM-241,385 binding was determined in the absence of any amiloride, while 0 % binding was determined as nonspecific binding of [^{3}H]ZM-241,385 assessed in the presence of 100 µM NECA (WT) or 10 µM ZM-241,385 (W246A$^{6.48}$). Values are means ± standard error of mean (SEM) of at least three independent assays performed in duplicate.



Compound	R	% [^{3}H]ZM-241,385 binding ± SEM to $hA_{2A}AR$ in the presence of 10 µM compound	
		WT	W246A$^{6.48}$
12a		68 ± 3	6.6 ± 0.3
12b		50 ± 3	2.1 ± 0.2
12c		92 ± 2	32 ± 1
12d		82 ± 1	11 ± 1
12e		67 ± 4	4.8 ± 1.0
12f		68 ± 2	5.8 ± 0.5

12g		72 ± 2	9.5 ± 0.8
12h		31 ± 1	-0.4 ± 0.1
12i		41 ± 2	5.4 ± 1.0
12j		51 ± 2	1.8 ± 0.5
12k		35 ± 2	1.0 ± 0.6
12l		27 ± 1	3.5 ± 0.2
12m		43 ± 2	2.2 ± 0.6
12n		66 ± 2	3.4 ± 0.8
12o		75 ± 2	4.0 ± 0.3
12p		63 ± 2	5.2 ± 0.3

The binding of the four most potent phenethyl derivatives, **12h**, **i**, **k**, and **l**, was characterized further by establishing their IC_{50} values in full curve assays for both receptors (Table 3). 4-Ethoxyphenethyl derivative **12l** had the highest potency at displacing [^{3}H]ZM-241,385 binding from the wild-type $hA_{2A}AR$, showing an IC_{50} value of 3.4 µM, which was lower than HMA (5.1 µM, Table 3). 4-Methoxyphenethyl derivative **12h** and dioxolyl derivative **12k** showed similar potencies compared to that of HMA, whereas 3-methoxyphenethyl derivative **12i** showed lower potency (8.1 µM) for radioligand displacement from the wild-type $hA_{2A}AR$. For the W246A$^{6.48}$ mutant $hA_{2A}AR$, compounds **12h**, **k**, and **l** showed increased potencies compared to that of HMA, whereas **12i** showed similar potency. The fold change in potency for W246A$^{6.48}$ compared to the wild-type receptor was 19-fold for HMA, approximately 35-fold for methoxyphenethyl derivatives **12h** and **i**, and approximately 80-fold for the dioxolyl **12k** and 4-ethoxyphenethyl **12l** derivatives.

Table 3. $hA_{2A}AR$ affinities of selected amiloride derivatives. IC_{50} values for the displacement of [^{3}H]ZM-241,385 binding to the $hA_{2A}AR$-WT and -W246A$^{6.48}$ by HMA **2** and phenethyl amiloride derivatives **12h**, **i**, **k**, and **l**. The concentration used of [^{3}H]ZM-241,385 was 2.5 nM. Nonspecific binding was determined in the presence of 100 µM NECA (WT) or 10 µM ZM-241,385 (W246A$^{6.48}$). Values are means ± SEM of at least three independent assays performed in duplicate.

	[^{3}H]ZM-241,385 displacement from $hA_{2A}AR$		
	WT	**W246A$^{6.48}$**	
	IC_{50} ± SEM (µM)	**IC_{50} ± SEM (µM)**	**Fold change to WT[a]**
HMA **2**	5.1 ± 0.2	0.27 ± 0.04	19
12h	4.4 ± 0.4	0.12 ± 0.01**	37
12i	8.1 ± 0.9*	0.25 ± 0.05	32
12k	5.6 ± 0.8	0.07 ± 0.01***	80
12l	3.4 ± 0.6*	0.05 ± 0.00***	76

a Change in fold IC_{50} of wild-type over W246A$^{6.48}$.

One-way ANOVA with Dunnett's post hoc test performed against HMA on log IC_{50} values, with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The effect of saturating concentrations of HMA and compounds **12h**, **i**, **k**, and **l** on the dissociation characteristics (residence time) of [^{3}H]ZM-241,385 from the wild-type and W246A$^{6.48}$ hA_{2A}AR was examined next (Table 4 and Figure 1). HMA had the largest effect on the dissociation velocity of [^{3}H]ZM-241,385 from the wild-type hA_{2A}AR, producing a 2.2-fold decrease in its residence time ($1/k_{off}$). Methoxyphenethyl derivatives **12h** and **i**, and 4-ethoxyphenethyl derivative **12l** decreased residence time by approximately 1.5-fold. In the case of dioxolyl derivative **12k**, the effect on the residence time was lost at the wild-type receptor. At the W246A$^{6.48}$ hA_{2A}AR, HMA, **12h**, and **12l** showed similar effects as those at the wild-type receptor, producing 1.9-, 1.3-, and 2.1-fold decreases in residence time, respectively. In contrast, the effect of 3-methoxyphenethyl derivative **12i** and dioxolyl derivative **12k** increased significantly at the W246A$^{6.48}$ mutant receptor, where 3.4- and 2.3-fold decreases in residence time of [^{3}H]ZM-241,385, respectively, were observed.

Table 4. Effect of amiloride derivatives on residence time of [^{3}H]ZM-241,385. Residence times of [^{3}H]ZM-241,385 dissociating from hA_{2A}AR-WT and -W246A$^{6.48}$ in the absence (control) or presence of 100 μM (WT) or 10 μM (W246A$^{6.48}$), i.e. saturating concentrations, of HMA **2** or amiloride derivatives **12h**, **i**, **k**, or **l**. The concentration of [^{3}H]ZM-241,385 was 2.5 nM. Nonspecific binding was determined in the presence of 100 μM NECA (WT) or 10 μM ZM-241,385 (W246A$^{6.48}$). Values are means ± SEM of at least three independent assays performed in duplicate.

	[^{3}H]ZM-241,385 dissociation from hA_{2A}AR Residence time (min)	
	WT	**W246A$^{6.48}$**
Control	61 ± 4	2.8 ± 0.4
+ HMA **2**	28 ± 2***	1.4 ± 0.2**
+ **12h**	44 ± 3**	2.0 ± 0.5
+ **12i**	42 ± 5*	0.8 ± 0.3**
+ **12k**	61 ± 5	1.2 ± 0.5*
+ **12l**	40 ± 1**	1.3 ± 0.3*

One-way ANOVA with Dunnett's post hoc test against control, with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

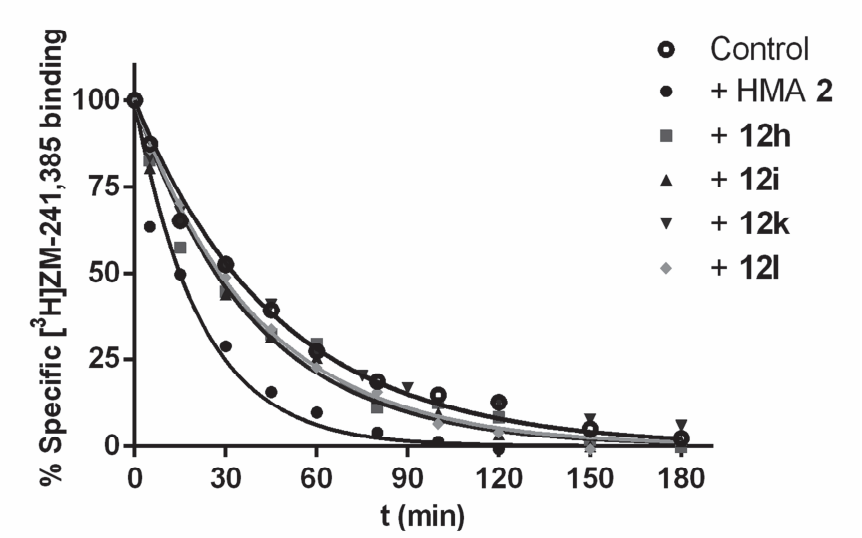


Figure 1. [^{3}H]ZM-241,385 dissociation from the hA_{2A}AR-WT in absence (control) or presence of 100 μM of HMA **2** or amiloride derivatives **12h**, **i**, **k**, or **l**. After allowing [^{3}H]ZM-241,385 to bind to its target, the dissociation was induced by addition of 100 μM NECA together with 100 μM of the respective compounds. Graph shows mean values of one representative dissociation experiment performed in duplicate.

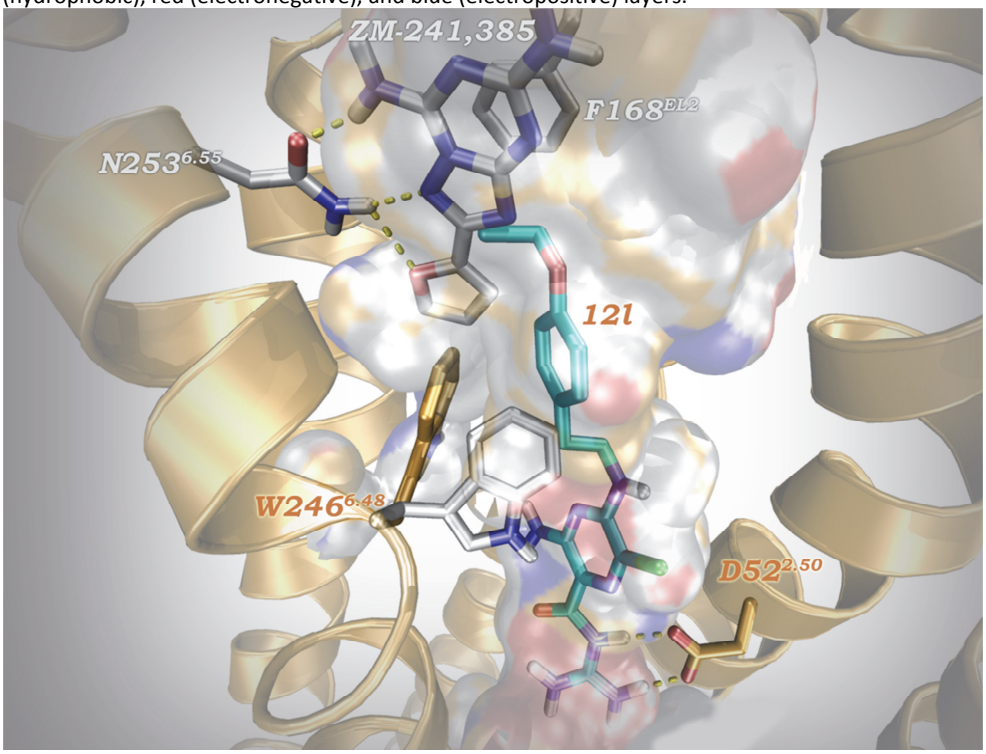
Docking study

The most potent amiloride derivatives, **12h**, **i**, **k**, and **l** were docked into the sodium ion binding site of the hA_{2A}AR X-ray cocrystal structure bearing orthosteric ligand ZM-241,385 (Figures 2 and S1-S3, PDB: 4EIY).[5] The cores of these amiloride derivatives were predicted to adopt binding poses similar to those of amiloride and HMA observed in Chapter 3 (Figure S1). The hydrogen bonding and salt bridge interactions with Asp52$^{2.50}$ and Thr88$^{3.36}$ were predicted similarly for the novel derivatives and HMA. Furthermore, **12h**, **i**, **k**, and **l** were predicted to occupy Trp246$^{6.48}$ position, forcing it to adopt a different rotameric position, as was observed previously for amiloride and HMA in Chapter 3.

However, the interactions with Trp246$^{6.48}$ were predicted to be different for each amiloride derivative. Whereas for HMA no π-π stacking was predicted, π-π stacking was predicted between Trp246$^{6.48}$ and the phenyl group of **12h**, **i**, and **k**, and a second π-π stacking interaction was predicted with the pyrazine core of **12i**. Furthermore, the phenethyl moieties of the novel derivatives were predicted to intrude a hydrophobic pocket not reached by the azepane moiety of HMA. Compared to HMA, the phenethyl

substitutions were predicted to be in a 4 Å range of three to four additional hydrophobic residues, specifically Phe168^{EL2}, Met177$^{5.38}$, Leu249$^{6.51}$ (**12h**, **i**, **k**, and **l**), and Ile274$^{7.39}$ (**12i** and **l**) (Figures S1 and S2). In addition, the dioxolyl and ethoxy substituents of compounds **12k** and **l** were predicted to be in the vicinity of Asn253$^{6.55}$ (Figure S2). Residues Phe168^{EL2}, Met177$^{5.38}$, Leu249$^{6.51}$, Asn253$^{6.55}$, and Ile274$^{7.39}$ are part of the orthosteric binding site, and their predicted vicinity to these residues indicates that **12h**, **i**, **k**, and **l** can intrude the orthosteric site and displace orthosteric ligand ZM-241,385 in a direct, competitive manner.

Figure 2. Docking of compound **12l** into the sodium ion binding site of wild-type hA_{2A}AR (PDB: 4EIY).[5] Compound **12l** is represented by cyan colored sticks, and ZM-241,385 in the orthosteric site is represented by grey sticks. Trp246$^{6.48}$ is represented by transparent white sticks (crystal structure position) and yellow sticks (proposed rotamer with **12l** docked in the sodium ion site). Oxygen and nitrogen atoms are represented in red and blue, respectively. The local protein backbone is represented by yellow ribbons, and relevant binding site confinements are indicated by white-grey (hydrophobic), red (electronegative), and blue (electropositive) layers.



Discussion

Amiloride and HMA act as allosteric modulators of several GPCRs,[10] including α_2 adrenergic receptors,[11] dopamine receptors,[12] and the gonadotropin-releasing hormone receptor.[13] Allosteric effects of both compounds have been extensively explored for adenosine receptors. Gao et al. demonstrated that HMA and a few other 5'-substituted amiloride derivatives increase the dissociation rate of antagonists from the adenosine A_1 and A_3 receptors, whereas amiloride itself was effective only at the adenosine A_1 receptor.[15] At the $hA_{2A}AR$, amiloride and its derivatives exhibited similar effects, where dissociation of radiolabeled antagonist [^{3}H]ZM-241,385 increases in their presence.[14] Amilorides supposedly bind in the sodium ion binding site of GPCRs. This has been visualized in Chapter 3 by docking amiloride and HMA into the high resolution X-ray crystal structure of the $hA_{2A}AR$.[5] Like sodium ions, the positively charged guanidinium moiety of amiloride and HMA made strong interactions with the carboxylate of Asp52$^{2.50}$. Furthermore, the presence of amiloride or HMA forced Trp246$^{6.48}$ to rotate away from its most stable position. This proposed binding mode was confirmed in the mutation study in Chapter 4, where the effects of amiloride and HMA were largely diminished by mutation D52A$^{2.50}$ but were increased by mutation W246A$^{6.48}$. The strong increase in potency of HMA compared to that of amiloride against the $hA_{2A}AR$ provided the impetus to investigate whether different substituents at the 5' position of amiloride might yield more potent amiloride derivatives. In the current study, a selection of 5'-substituted amilorides was tested for binding to the wild-type and W246A$^{6.48}$ mutant $hA_{2A}AR$ (Table 1).

Carboxylate derivatives **3** and **5** and tetrazole **7** showed minimal or no displacement of [^{3}H]ZM-241,385 binding from either receptor. Apparently, amiloride derivatives carrying a negative charge on an extended 5'-substituent do not bind in the sodium ion pocket. Indeed, 5' substituents are predicted to enter a hydrophobic pocket, as suggested by the proposed binding modes of HMA and **12h**, **i**, **k**, and **l** (Figures S1 and S2). Most of the other amilorides were more or less active at the W246A$^{6.48}$ mutant $hA_{2A}AR$, indicating at least some affinity of these compounds for the sodium ion site. The most potent derivative at both the wild-type and mutant $hA_{2A}ARs$ in the preliminary series was phenethyl derivative **12**. This derivative was the starting point for the synthesis of novel derivatives **12a**-**p** carrying substituents on the phenyl moiety (Table 2). The four most potent derivatives to emerge for the wild-type receptor were **12h**, **i**, **k**, and **l**, of which 4-ethoxyphenethyl

derivative **12l** proved to be more potent than HMA (Table 3). Differences in potency shifts between the wild-type and W246A$^{6.48}$ mutant hA_{2A}AR ranged from 19-fold for HMA to 80-fold for **12k**. Together with the predicted binding modes, in which Trp246$^{6.48}$ is forced to rotate away from its most stable position, as observed previously for amiloride and HMA in Chapter 3 (Figures 2 and S3), these potency shifts support the binding of these ligands in the sodium ion site. The bulkiest compounds, dioxolyl derivative **12k** and 4-ethoxyphenethyl derivative **12l**, benefited the most from the absence of the bulky tryptophan residue, which suggests increased steric clash between Trp246$^{6.48}$ and bulkier 5' substituents.

Trp246$^{6.48}$ separates the sodium ion binding pocket from the orthosteric binding pocket, which are adjacent.[5] The proposed binding modes in the docking study predicted that the elongated phenethyl substituents of the amiloride derivatives are in close proximity to at least four amino acids that make up the orthosteric pocket (Figures 2 and S1-S3), which makes it likely that they protrude into the orthosteric pocket and enter in a direct competition with orthosteric ligands. This would change the allosteric mechanism of these amiloride derivatives from a noncompetitive to a more competitive nature. To investigate whether this was the case, the four most potent phenethyl-amiloride derivatives, **12h**, **i**, **k**, and **l**, were tested for their influence on the dissociation characteristics of the orthosteric ligand [^{3}H]ZM-241,385 from the wild-type hA_{2A}AR (Table 4). The noncompetitive allosteric effect of HMA was evident as it decreased the residence time of [^{3}H]ZM-241,385 significantly at the wild-type receptor, which is in agreement with previous studies (Gao et al.[14] and Chapter 3). Compounds **12h**, **i**, and **l** were more competitive in nature, as illustrated by their smaller effects on the dissociation characteristics of the orthosteric ligand. Interestingly, compound **12k** did not influence the dissociation of [^{3}H]ZM-241,385 at all, which suggests that the displacement of the orthosteric ligand by **12k** is entirely due to direct competition between the two ligands.

The position of Trp246$^{6.48}$ at the interface of the orthosteric and allosteric sites of the hA_{2A}AR suggests that this amino acid influences the nature of the allosteric mechanism of amilorides. Indeed compounds **12i** and **k** showed quite different effects on the dissociation of [^{3}H]ZM-241,385 from the W246A$^{6.48}$ mutant compared to the wild-type receptor. These two compounds became noncompetitive allosteric modulators in the absence of the bulky tryptophan side chain, as their effect on the dissociation

characteristics of [^{3}H]ZM-241,385 increased significantly. For **12k**, the effect was remarkable, showing no effect at the wild-type receptor while producing a 2.3-fold decrease in the residence time of [^{3}H]ZM-241,385 at the mutated receptor. This suggests that the presence of Trp246$^{6.48}$ 'guides' the methoxy and dioxolyl substituents on the phenethyl moieties of **12i** and **k** toward the orthosteric site, inducing their competitive effect.

Conclusions

We demonstrated that it is possible to extend amiloride with substituents at the 5' position to produce amiloride derivatives with similar or even higher potencies than HMA at the wild-type hA_{2A}AR. Similar to amiloride and HMA, all novel amiloride derivatives showed significantly higher potencies at the W246A$^{6.48}$ mutant hA_{2A}AR, implying that Trp246$^{6.48}$ hinders the binding of these amiloride derivatives. HMA showed the largest allosteric effect on [^{3}H]ZM-241,385 dissociation, whereas the most potent novel amilorides showed reduced or no effects on this dissociation process. This indicates that these novel amilorides engage in a more direct competition with the orthosteric ligand, and it can be hypothesized that they do so by intrusion into the orthosteric pocket. The striking differences in effect on the dissociation of [^{3}H]ZM-241,385 between the wild-type and W246A$^{6.48}$ hA_{2A}ARs imply that Trp246$^{6.48}$ influences the nature of the allosteric interaction by amilorides. The well-conserved sodium ion pocket and the effects of amilorides and HMA shared among a multitude of GPCRs suggest a general mechanism of their interaction with the superfamily of GPCRs.

Supplemental information

Supplemental information includes full experimental procedures for synthesis of compounds **12a-p**, including NMR and HPLC data, 2D interaction maps of docking of HMA **2** and **12h**, **i**, **k**, and **l (**Figures S1 and S2), 3D representation of docking of **12h**, **i**, **k**, and **l** (Figure S3), and SMILES strings and binding and inhibition data for **1**-**18** and **12a**-**p** in (CSV). It can be found with this article online at dx.doi.org/10.1021/ acs.jmedchem.6b00142

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