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Medetomidine analogs as selective agonists for the human α_2-adrenoceptors

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Abstract

α_2-Adrenoceptor (AR) agonists have therapeutic applications in a variety of diseases. Medetomidine, an α_2-AR agonist, belongs to 4-substituted imidazole class of compounds and is highly selective for the α_2-AR. The benzylic methyl group of medetomidine and naphthalene imidazole was proposed to interact with rat brain α_2-ARs via a methyl binding pocket in a manner analogous to its presence in α-methyl norepinephrine. A series of derivatives containing hydrophilic and hydrophobic substituents, as well as chiral and conformationally rigid analogs were used. In current binding and functional studies using human α_2-AR subtypes expressed in Chinese hamster ovary cells, optimal interactions were observed with the presence of the methyl group on the benzylic carbon atom of naphthyl imidazole. Data obtained with various analogs have demonstrated that size, electronegativity, lipophilicity, chirality and conformational flexibility of the substituents at the carbon bridge of naphthyl imidazole are important factors for interaction of the imidazole class of ligands with these α_2-AR subtypes. Taken collectively, the results obtained support the existence of the methyl binding pocket for optimal ligand receptor binding interactions in human α_2-AR subtypes. Further, the results also suggest that, additional modifications of medetomidine and naphthyl methyl imidazole at the benzylic carbon atom, and/or on the aromatic and imidazole ring systems could provide insights into the chemical requirements for optimizing α_2-AR subtype selectivity. This could eventually lead to the discovery of promising compounds for the evaluation of the physiological importance of the three α_2-AR subtypes.

Keywords: α_2-Adrenoceptors; Imidazole; Medetomidine; Methyl pocket; Radioligand binding assay; cAMP

1. Introduction

α_2-Adrenoceptors (ARs) are membrane-bound receptors located throughout the body on neuronal and non-neuronal tissues, where they mediate a diverse range of responses to the endogenous catecholamines, norepinephrine, and epinephrine [1]. α_2-AR agonists have therapeutic applications in a variety of diseases, including hypertension, angina pectoris, congestive heart failure, cardiac arrhythmia, asthma, depression, prostatic hypertrophy, and glaucoma. Many of these drug effects are mediated through activation of heterogenous α_2-AR populations, and limit the therapeutic usefulness of this class of drugs.

The α_2-ARs have been cloned and proposed to consist of seven transmembrane-spanning domains that are connected by three extracellular and three intracellular loops, with the amino terminus being extracellular and the carboxy terminus intracellular. The 3D structure of the transmembrane-spanning α-helices of the α_2-AR might resemble the folding of bacteriorhodopsin, a membrane protein whose 3D structure has been obtained by cryomicroscopy [2]. Site-directed mutagenesis combined with molecular modeling has predicted several potential sites for the interaction of catecholamines with the α_2-ARs, which agrees with the 3-point attachment, as suggested by

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Abbreviations: CHO, Chinese hamster ovary; HEK, human embryonic kidney.

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the Easson–Stedman hypothesis [3,4]. However, addition of a methyl group to norepinephrine, i.e. 1*R*,2*S*-(−)-α-methyl norepinephrine has enhanced α_2-AR selectivity. Based on this α_2-AR selectivity for α-methyl norepinephrine, Ruffolo *et al*. [5] suggested a fourth site of interaction for the α-methyl group of 1*R*,2*S*-(−)-α-methyl norepinephrine to the α_2-ARs. Molecular modeling has suggested that the α-methyl group of 1*R*,2*S*-(−)-α-methyl norepinephrine is surrounded by the side chain of four amino acids which form a lipophilic cavity and has been called the ''methyl pocket'' [6]. Also, the benzylic methyl group of medetomidine, a 4-substituted imidazole, and the α-methyl group of (−)-α-methyl norepinephrine were superimposable, indicating that these methyl groups may fit into the same pocket at the α_2-ARs [6].

The overall objective of the present investigation was to establish whether the methyl pocket hypothesis could be valuable to identify novel α_2-AR subtype-selective ligands. The aims of this study were to determine the importance of the methyl group of medetomidine and of closely related naphthyl imidazole analogs on binding to the human α_2-AR subtypes, to evaluate the influence of stereochemistry and structural modification at the carbon bridge between the naphthalene and imidazole rings of naphthyl imidazole analogs on α_2-AR affinity, and to determine functional potency of selected imidazole analogs on the human α_2-ARs. In order to achieve these goals, chiral and conformationally rigid analogs, and compounds containing a variety of lipophilic and hydrophilic groups placed at the carbon bridge of the naphthyl imidazole analogs were prepared and biological activities were characterized on the human α_2-AR subtypes expressed in Chinese hamster ovary (CHO) cells. The binding affinities on the three α_2-AR subtypes was compared with the binding affinities obtained on the human α_{1A}-AR subtype. For a series of naphthyl analogs, the functional potencies for inhibition of forskolin-induced cAMP elevations in the α_2-ARs were evaluated and compared to their binding affinities.

2. Materials and methods

2.1. Materials

All cell culture reagents were obtained from Life Technologies. Human α_{2A}-, α_{2B}- and α_{2C}-AR subtypes expressed in CHO cells were obtained from Dr. Marc Caron, Dr. Robert Lefkowitz and Dr. Stephen Liggett. The human α_{1A}-AR subtype expressed in human embryonic kidney (HEK) cells was obtained from Dr. Kenneth Minneman. All of the imidazole analogs were provided by Dr. Duane D. Miller. The synthesis of these compounds have been reported previously [6–8]. The compounds were dissolved in water or dissolved in a 1:5 mixture of DMSO:-water. Stock solutions of 10 mM were prepared fresh daily and diluted in water to appropriate concentrations for these studies. The compounds were assumed to be stable since they were prepared fresh daily and data obtained were highly reproducible. [^{3}H]Rauwolscine and [^{3}H]prazosin were obtained from NEN Life Science Products. The luciferase reporter gene construct (6 CRE-LUC, pADneo2-C6-BGL) [9] was provided by Dr. A. Himmler. All other chemicals were obtained from Sigma Chemical Company.

2.2. Cell culture

CHO cells stably expressing human α_{2A}-, α_{2B}- and α_{2C}-AR subtypes were grown in 150 cm^2 Corning flasks with Ham's F12 media supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 unit/mL), streptomycin (100 μg/mL) and geneticin (final concentration was 80 μg/mL). The flasks were incubated at 37° (5% CO_2). Media was changed every 48 hr until the cells were confluent. Upon confluency, the cells were detached by addition of 0.05% trypsin EDTA for 3–5 min.

HEK cells stably expressing human α_{1A}-AR subtype was grown in 150 cm^2 Corning flasks with Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 unit/mL), streptomycin (100 μg/mL) and geneticin (final concentration was 80 μg/mL). The flasks were incubated at 37° (5% CO_2). Media was changed every 48 hr until the cells were confluent. Upon confluency, the cells were detached by gentle scraping.

2.3. Radioligand binding assays

Radioligand binding studies were performed in CHO cells expressing human α_{2A}-, α_{2B}- and α_{2C}-AR subtypes and HEK cells expressing human α_{1A}-AR subtype. The detached cells were washed, centrifuged with Tris–EDTA buffer, pH 7.4; containing 50 mM Tris, 20 mM di-sodium EDTA and 154 mM NaCl, in which they were finally suspended. The competition binding assays were performed in duplicate by incubating 50,000 cells with [^{3}H]rauwolscine and [^{3}H]prazosin for the α_2- and α_{1A}-AR, respectively, and varying concentrations of the compounds under investigation for 1 hr in a water bath at 37°. Non-specific binding was defined by addition of 10 μM yohimbine and 10 μM phentolamine for the α_2- and α_{1A}-AR, respectively. Phentolamine produced the same maximal displacement as that of yohimbine suggesting that either compound is appropriate for the measurement of non-specific binding. Non-specific binding for both phentolamine and yohimbine was about 5% of the total binding. Cell suspensions were filtered using Whatman GF/B glass fiber filters using a Brandel model 12-R cell harvester. The filter discs were washed three times with 5 mL of Tris–EDTA buffer (pH 7.4 at 4°). The radioactivity was determined using a Packard Tri Carb 2900TR liquid scintillation counter. The displacement curves were generated using

Graph Pad Prism software (Graph Pad Software Inc.) and the K_i values of the competing ligands were determined using the equation of Cheng and Prusoff [10]. The percent specific binding was determined by dividing the difference between total bound (DPM) and non-specific bound (DPM) by the total bound (DPM).

Scatchard analyses for radioligands were determined using varying concentrations of [^{3}H]rauwolscine and [^{3}H]prazosin, alone or in the presence of fixed concentrations of yohimbine or phentolamine. The specific binding was established at each concentration and plotted as bound ligand vs. bound/free ligand and the corresponding B_{max} and K_D values calculated on each human adrenoceptor subtype.

The experimentally determined K_D (nM) and B_{max} (nmol/mg) values (mean ± SEM) of the radioligands on the AR subtypes were: [^{3}H]rauwolscine: $\alpha_{2A} = 1.14 \pm 0.15$ and 0.21 ± 0.03; $\alpha_{2B} = 0.63 \pm 0.07$ and 0.19 ± 0.07; $\alpha_{2C} = 0.28 \pm 0.05$ and 0.07 ± 0.003 in CHO cells; and [^{3}H]prazosin: $\alpha_{1A} = 0.20 \pm 0.02$ and 0.22 ± 0.02; $\alpha_{1B} = 0.11 \pm 0.01$ and 0.38 ± 0.01; $\alpha_{1D} = 0.17 \pm 0.07$ and 0.11 ± 0.04, respectively in HEK cells [11].

2.4. Cyclic AMP response element-luciferase reporter gene assay

In order to verify that the observed binding affinities of medetomidine and its analogs correlate with the functional responses in these α_2-AR subtypes, we examined the agonist effects of medetomdine and selected analogs for their ability to reverse forskolin-induced cAMP on CHO cells expressing the human α_{2A}-, and α_{2C}-AR subtype. Cyclic AMP levels in these cells were determined by measuring changes in luciferase activity. The cell-based cAMP response element-luciferase reporter gene assay (CRE-LUC) was used as described previously by Vansal and Feller [12]. Preliminary studies were done to show that the 4 hr time period was appropriate for the measurement of α_2-AR subtype-dependent luciferase activity changes in forskolin response in the presence of drugs used in these experiments. The compounds were tested under the following conditions: the 6 CRE-LUC plasmid was transiently transfected into CHO cells expressing the α_2-AR subtype using electroporation at 150 V, 70 ms, single pulse. The transfected cells were plated at a density of 50,000 cells/200 μL per well in a 96-well microtiter plate and allowed to grow for 20 hr. The compounds under investigation for agonist activity were added directly to the media 20 min prior to the addition of forskolin and allowed to incubate for 4 hr. Subsequently, the media was aspirated, the cells lysed and the luciferase activity determined using a Topcount (Packard Instrument Company) luminometer after addition of luciferin. Data were analyzed using Graph Pad Prism software and expressed as a mean ± SEM. The response produced by forskolin (5 μM) in each experiment was used as 100%.

2.5. Molecular modeling and NMR studies for selected imidazole ligands

All spectra were acquired at 23° and 500 MHz on a Varian Inova-500 spectrometer using a 5-mm HCN triple resonance probe. Both proton and carbon chemical shifts were referenced to the residual solvent peak of DMSO (2.49 ppm for proton and 40 ppm for carbon). For 2D rotational nuclear Overhauser effect spectroscopy (ROESY) measurements, a total of 512 free-induction decays (fids) were recorded for the indirect dimension, with a 2 s recycle delay, 300 ms mixing time. The TRIAD NMR package within the Sybyl software was used for data processing and analysis. Peaks in the ROESY spectra were assigned and integrated using TRIAD standard functions. MARDIGRAS was then used to generate distance constraints for the *E*-ethylene and *Z*-ethylene using these peak integrals. The resulting constraints were then examined to ensure, that the error in distances conformed to established errors for nuclear Overhauser effect (NOE) constraints wherein; $x < 2.5$ Å was ±0.1 Å; $x \leq 3.0$ Å was ±0.2 Å; $x \leq 3.5$ Å was ±0.3 Å; and $x \geq 3.5$ was ±0.4 Å. A four-step simulated annealing using 1 fs time steps and the constraints generated by MARDIGRAS was performed on 28 as follows: (1) 1 ps dynamics at 300 K; (2) 1 ps heating to 500 K; (3) another 1 ps heating phase to 700 K; (4) a 1 ns equilibration to 500 K. Additional parameters included the Tripos force field with Gasteiger–Hückel charges, an 8 Å non-bonding cutoff, and distance-dependent dielectric constant function. The experimentally obtained NOE distance constraints were applied during all steps of the dynamics runs. The molecular geometry was sampled at 1000 fs intervals during phase (1) of the dynamics runs and once during the heating and cooling periods. A total of 1007 conformations were collected during for further analysis and these were subjected to 20 dynamics simulations each to obtain average conformations. These average conformations were then minimized with a gradient tolerance of 0.005 kcal/mol Å without defined aggregates or experimental NOE distance constraints to obtain the final average conformations.

2.6. Data accumulation and statistical analyses

For binding studies in cell lines, varying concentrations of each drug/ligand (ranging from 10^{-11} to 10^{-4} M) were added in duplicate within each experiment, and the individual molar inhibitory concentration 50 (IC_{50}) values were determined using Graph Pad Prism software. The K_i values of each ligand were determined according to the equation described by Cheng and Prusoff [10], and final data presented as $pK_i \pm$ SEM of $N \geq 4$ experiments. The concentration-dependent reversal of forskolin on luciferase activity changes in CHO cells by medetomidine and selected analogs were determined as molar effective concentration 50 (EC_{50}) values ± SEM of $N = 3$ experiments.

Statistical analysis of comparisons between means was done by use of the ANOVA and Student's *t* test at the 5% level of significance. When more than two means were compared, differences between means of binding affinities and functional responses for individual ligands on the α-AR subtypes were done by ANOVA. If a significant *F* value was found, Tukey's *post hoc* analysis test for multiple comparisons was employed to identify differences among groups [13].

3. Results

In order to obtain a better understanding and to support the methyl pocket hypothesis, the binding affinity of medetomidine and closely related naphthyl imidazole analogs were compared on the human α_{1A}- and α_2-ARs. Structures of all the naphthyl imidazole analogs are provided in Fig. 1. pK_i values for all the compounds tested have been presented in Tables 1 and 2; while the K_i values

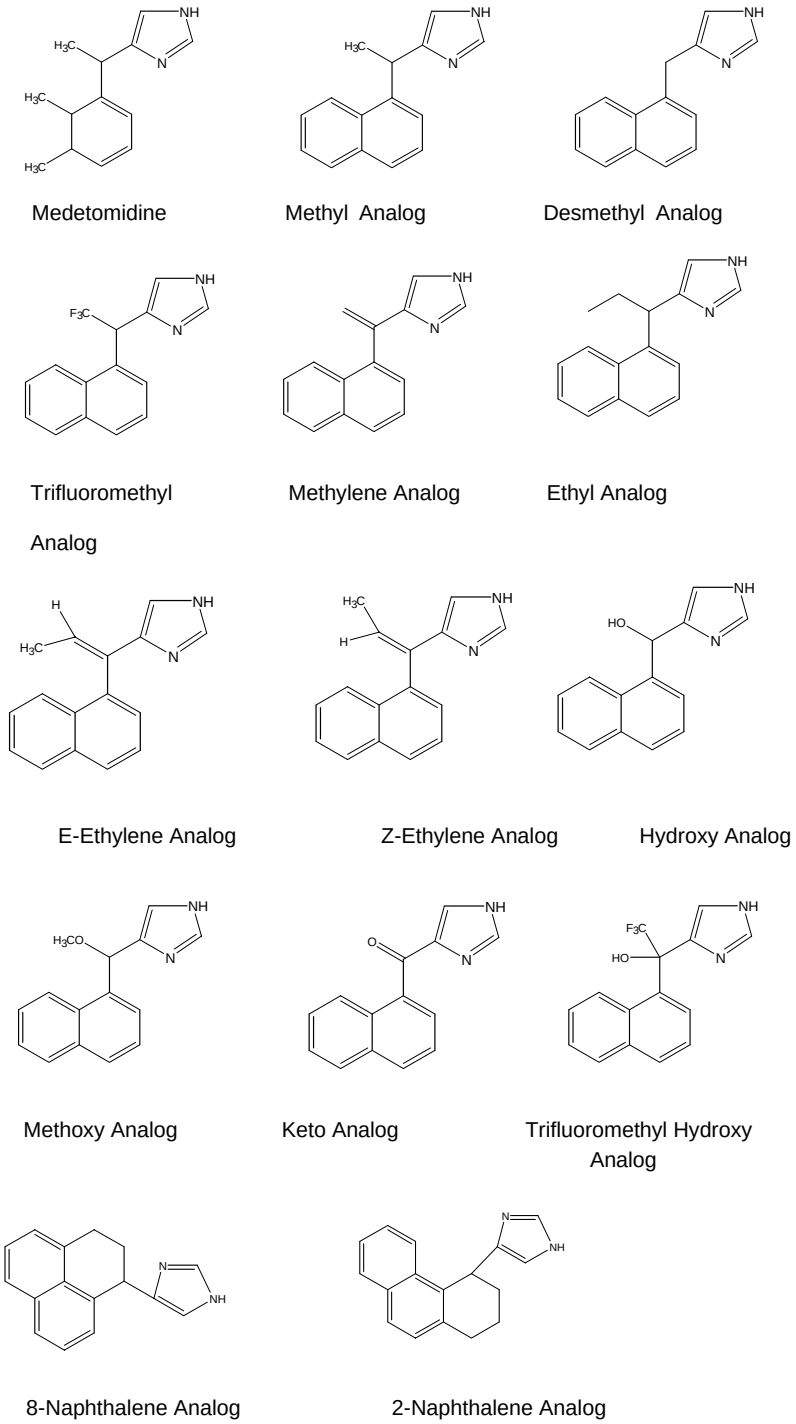

Fig. 1. Structures of medetomidine and napthyl imidazole analogs.

Table 1
Binding affinities (pK_i values) of medetomidine and naphthyl imidazole analogs on human α_{1A}-, α_{2A}-, α_{2B}- and α_{2C}-ARs expressed in HEK and CHO cells[a]

Compound	Adrenergic receptor subtype (pK_i ± SEM)			
	α_{1A}-AR	α_{2A}-AR	α_{2B}-AR	α_{2C}-AR
Medetomidine	5.60 ± 0.02	7.56 ± 0.06[b]	7.50 ± 0.03[b]	7.40 ± 0.08[c]
Methyl analog	6.10 ± 0.10	7.87 ± 0.05[d]	7.40 ± 0.07	7.70 ± 0.02[d]
(−)-Methyl isomer	6.35 ± 0.03	7.81 ± 0.04[d]	7.45 ± 0.07	7.75 ± 0.06[d]
(+)-Methyl isomer	5.95 ± 0.24	7.79 ± 0.02[d]	7.39 ± 0.03	7.70 ± 0.05[d]
Desmethyl analog	7.00 ± 0.02	7.56 ± 0.01[b]	7.40 ± 0.11[b]	7.40 ± 0.04[b]

Structures of compounds are shown in Fig. 1 and data is obtained from displacement curves shown in Fig. 2.

[a] [^{3}H]Prazosin and [^{3}H]rauwolscine were used as the radioligands in the equilibrium competition radioligand binding assays for the α_1- and α_2-ARs, and non-specific binding was measured in the presence of 10 μM of yohimbine and 10 μM of phentolamine, respectively. pK_i value = −log K_i (K_i was calculated according to the Cheng–Prusoff equation) and the data are the mean ± SEM of N = 4–6. Statistical analysis was carried out by one-way ANOVA followed by Tukey's test.

[b] Indicates mean pK_i value is significantly different from the mean pK_i value for the α_{1A}-AR subtype.

[c] Indicates mean pK_i value is significantly different from the mean pK_i values for the α_{1A}- and α_{2A}-AR subtypes.

[d] Indicates mean pK_i value is significantly different from the mean pK_i values for the α_{1A}- and α_{2B}-AR subtypes.

and fold-differences between compounds are presented below. The rank order of potency (K_i values) of medetomidine and the methyl naphthyl imidazole for the four subtypes of receptors tested are $\alpha_{2A} = \alpha_{2B} = \alpha_{2C} \gg \alpha_{1A}$ (Fig. 2A and B). The data in Table 1 demonstrate that medetomidine and methyl naphthyl imidazole possess greater affinity for the α_2-ARs than the α_{1A}-AR (pK_i values are presented in Table 1), and further, that they are non-selective ligands for the α_2-AR subtypes. In contrast, the desmethyl naphthyl imidazole analog, which lacks the α-methyl group at the benzylic carbon atom, shows almost overlapping competition curves for α_{1A}-AR and the three α_2-AR subtypes (Fig. 2C). Thus, the results on the binding studies for the 4-substituted imidazoles support the importance of the methyl group in providing α_2- vs. α_1-AR selectivity.

To evaluate the influence of stereochemistry of the methyl group at the carbon bridge, the racemate and stereoisomers of methyl naphthyl imidazole were examined on the human α_{1A}- and the α_2-ARs. The racemate and *S*-(+)- and *R*-(−)-isomers of methyl naphthyl imidazole had binding affinities that were similar to those of medetomidine on the four α-AR subtypes; and in addition, these compounds were at least 13-fold more selective for the α_2-AR subtypes vs. the α_{1A}-AR (Table 1). It is notable that the two stereoisomers and racemate of methyl naphthyl imidazole also exhibited the same binding affinities on these four α-AR subtypes. Further, to determine conformational preferences of methyl naphthyl imidazole, two novel conformationally restricted analogs of methyl naphthyl imidazole (Fig. 1), which incorporated the benzylic methyl group at either the 8-position or 2-position

Table 2
Binding affinities (pK_i values) of naphthyl imidazole analogs on human α_{1A}-, α_{2A}-, α_{2B}- and α_{2C}-ARs expressed in HEK and CHO cells[a]

Compound	Adrenergic receptor subtype (pK_i ± SEM)			
	α_{1A}-AR	α_{2A}-AR	α_{2B}-AR	α_{2C}-AR
Trifluoromethyl	3.60 ± 0.28	4.78 ± 0.06[c,d]	4.71 ± 0.03[c]	5.30 ± 0.10[c,f]
Methylene	5.64 ± 0.05	6.88 ± 0.06[e]	6.34 ± 0.03[c]	5.74 ± 0.03[c,f]
Ethyl	5.72 ± 0.05	6.80 ± 0.08[e]	6.32 ± 0.04[c]	6.59 ± 0.05[c,f]
E-Ethylene	6.12 ± 0.02	5.33 ± 0.05[e]	4.72 ± 0.05[c]	6.10 ± 0.03[f]
Z-Ethylene	4.69 ± 0.04	5.90 ± 0.04[e]	5.37 ± 0.04[c]	5.81 ± 0.05[e]
Hydroxy	4.79 ± 0.05	6.00 ± 0.04[e]	5.60 ± 0.06[c]	4.98 ± 0.07[c,f]
Methoxy	4.39 ± 0.05	5.12 ± 0.04[e]	5.03 ± 0.06[c]	5.11 ± 0.05[c]
Keto	5.77 ± 0.05	4.85 ± 0.13[c]	3.33 ± 0.60[c]	4.43 ± 0.07[e]
Trifluoromethyl hydroxy	<4.00[b]	3.66 ± 0.40	3.52 ± 0.50	4.20 ± 0.11
8-Naphthalene	7.19 ± 0.07	7.44 ± 0.04[e]	6.66 ± 0.10	7.14 ± 0.02[f]
2-Naphthalene	5.74 ± 0.05	5.43 ± 0.05[c]	5.39 ± 0.08[c]	5.60 ± 0.21

Structures of compounds are shown in Fig. 1.

[a] [^{3}H]Prazosin and [^{3}H]rauwolscine were used as the radioligands in the equilibrium competition radioligand binding assays for the α_1- and α_2-ARs, and non-specific binding was measured in the presence of 10 μM of yohimbine and 10 μM of phentolamine, respectively. pK_i value = −log K_i (K_i was calculated according to the Cheng–Prusoff equation) and the data are the mean ± SEM of N = 4–6.

[b] Maximum % inhibition was 27 at 0.1 mM. Statistical analysis was carried out by one-way ANOVA followed by Tukey's test.

[c] Indicates mean pK_i value is significantly different from the mean pK_i value for the α_{1A}-AR subtype.

[d] Indicates mean pK_i value is significantly different from the mean pK_i values for the α_{1A}- and α_{2A}-AR subtypes.

[e] Indicates mean pK_i value is significantly different from the mean pK_i values for the α_{1A}- and α_{2B}-AR subtypes.

[f] Indicates mean pK_i value is significantly different from the mean values for the α_{2A}- and α_{2B}-AR subtypes.

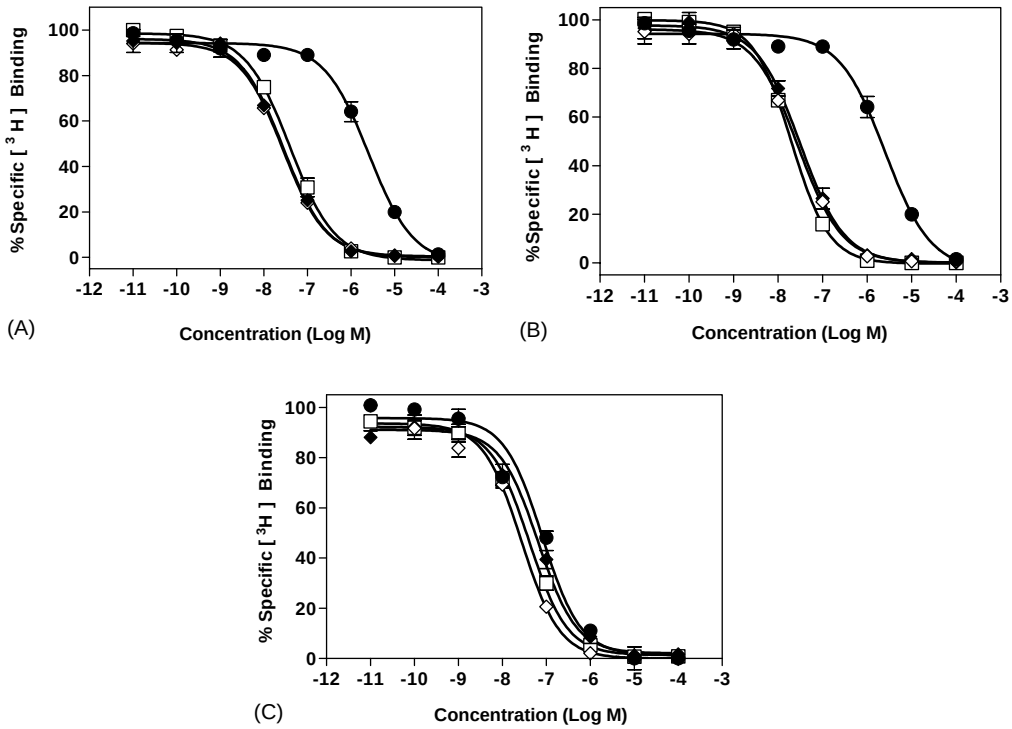

Fig. 2. Binding displacement curves of (A) medetomidine and (B) the methyl and (C) desmethyl analogs of naphthyl imidazole for human α_{1A}-, α_{2A}-, α_{2B}- and α_{2C}-ARs expressed in HEK and CHO cells: data are expressed as mean ± SEM (N = 4–6 experiments) and pK_i ± SEM are presented in Table 1. Key: α_{2A}, (◇); α_{2B}, (◆); α_{2C}, (□); and α_{1A}, (●).

of the naphthalene ring, were evaluated for binding to these receptor subtypes. The two conformationally rigid molecules exhibited significantly different binding affinities (Table 2). While the 8-naphthalene analog had higher affinities (>12-fold) for the α_2-ARs, as compared to those of the 2-naphthalene analog; it also displayed high affinity at the α_{1A}-AR and thus a loss of α_2- vs. α_1-AR subtype selectivity. In this regard, the 2-naphthalene exhibited relatively similar affinities for the α_2- and α_{1A}-AR subtype.

To gain a better understanding of the structural requirements for the methyl binding pocket for ligands, and to test whether modifying the methyl group could be used as an approach to obtain highly potent and α_2-AR subtype-selective ligands, we tested a series of naphthyl imidazole analogs with varying substituents at the benzylic carbon position (Fig. 1). The binding data for these analogs on the human α_2-AR subtypes are presented in Table 2. As compared to methyl naphthyl imidazole, all of the naphthyl imidazole analogs showed lower binding affinities. For the imidazole analogs the rank order potency (K_i, nM) for the human α_{2A}-, α_{2B}- and α_{2C}-AR subtypes was; naphthyl methyl analog (14, 40, and 20) ≥ medetomidine (27, 32, and 40) = desmethyl (28, 40, and 40) > hydroxy (1000, 2500, and 10,500) > trifluoromethyl analog (16,600, 19,500, and 5000), respectively. These findings demonstrate the importance of the methyl group for optimal binding to the α_2-AR subtypes. Among the α_2-AR subtypes the *E*-ethylene analog showed a 6- and 24-fold greater affinity for the α_{2C}- vs. α_{2A}- and α_{2B}-AR; the trifluoromethyl analog showed a 3- and 4-fold greater affinity for the α_{2C}-AR subtype; and the hydroxy analog showed a 3- and 10-fold higher affinity for the α_{2A}-AR subtype vs. the α_{2B}- and α_{2C}-AR. The trifluoromethyl, methylene, ethyl, *Z*-ethylene and hydroxy analogs gave higher affinities for the α_2-AR subtypes vs. the α_{1A}-AR subtype. Di-substitution at the benzylic carbon atom of naphthyl imidazole with trifluoromethyl and hydroxyl groups (trifluoromethyl hydroxy, Table 2), produced the greatest reduction in affinity for the α_2-AR subtypes, and the affinities were at less 6-fold lower than those of the corresponding monosubstituted trifluoromethyl and hydroxy analogs on these subtypes. Thus, the modification of the benzylic carbon substituent of methyl naphthyl imidazole produced compounds with reduced affinities, and small differences in affinities among the three α_2-AR subtypes and selectivities for α_2-AR subtypes vs. the α_{1A}-AR subtype.

In order to verify that the observed binding affinities of medetomidine and its analogs correlate with the functional responses in the α_2-ARs, the agonist effects of medetomidine and selected analogs were examined for their abilities to reverse forskolin-induced cAMP responses on CHO cells expressing the human α_{2A}- and α_{2C}-AR subtypes. Cyclic AMP changes were measured using the 6 CRE-luciferase reporter gene assay (CRE-LUC) [8]. In cells expressing the α_{2A}- and α_{2C}-AR subtype and transfected with reporter plasmid, the inhibition of forskolin-induced cAMP elevations by each agonist was determined

at varying concentrations (10^{-12} to 10^{-5} M). The rank order potency (EC_{50}, nM) for naphthyl imidazole analogs and medetomidine on the α_{2A}-AR subtype was medetomidine (0.45) > methyl (1.62) > desmethyl (4.88) > trifluoromethyl (maximum percent inhibition was 24% at 10^{-5} M). The rank order potency (EC_{50}, nM) for these naphthyl imidazole analogs vs. medetomidine on the α_{2C}-AR subtype was medetomidine (0.95) ≥ desmethyl (1.09) > methyl (4.57) > trifluoromethyl (maximum percent inhibition was 25% at 10^{-5} M). The rank order potencies of these analogs in functional studies were similar to those in binding studies for these two subtypes. Although the extent of medetomidine reversal of forskolin stimulated cAMP was not equal for the methyl, desmethyl and medetomidine derivatives, their EC_{50} values were very comparable. For the methyl naphthyl imidazole isomers, the *S*-(+)-isomer was much more potent than the *R*-(−)-isomer for reversing the action of forskolin on the α_{2A}- and α_{2C}-subtypes. The EC_{50} values (nM) for *S*-(+)-methyl naphthyl imidazole vs. the *R*-(−)-isomer was 0.81 and 1445 for the α_{2A}-subtype and 2.2 and 575 for the α_{2C}-subtype, respectively (Fig. 3, Table 3). The corresponding functional agonist potency differences for these isomers on these two subtypes were 1783- and 261-fold, respectively. This evidence also suggests that the *R*-(−)-enantiomer acts as a partial agonist, since binding affinities of the isomers on this AR subtype were the same (Table 1).

The receptor affinities of these compounds suggested that unique structural elements and geometries were, in part, responsible for the observed selectivity. To gain insight into the geometrical requirements, NMR and molecular modeling studies were conducted on the *E*-ethylene and *Z*-ethylene analogs. These compounds were selected due to the rigidity imparted by the inclusion of the ethylene functionality. The 2D ROESY studies provide six inter-proton constraints for the *E*-ethylene and four for the *Z*-ethylene, which were utilized in simulated annealing

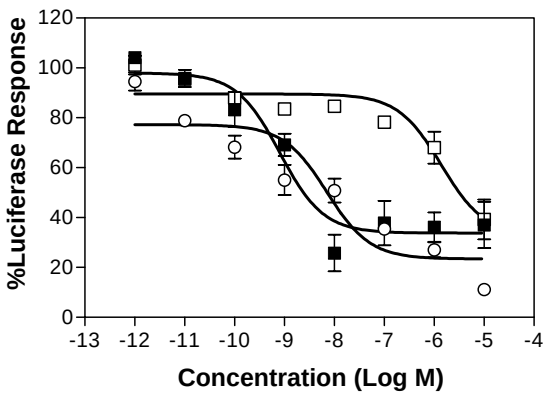

Fig. 3. Concentration-dependent reversal of forskolin-induced cAMP elevations by racemate and the *R* and *S* isomers of methyl naphthyl imidazole on human α_{2A}-AR. Data are expressed as mean ± SEM (N = 4–5 experiments) and pEC_{50} ± SEM are presented in Table 3. Key: *racemic*-methyl naphthyl imidazole, (○); *S*-(+)-methyl naphthyl imidazole, (■); *R*-(−)-methyl naphthyl imidazole, (□).

Table 3
Concentration-dependent effects of medetomidine and naphthyl imidazole analogs for the reversal of forskolin-induced cAMP elevations on α_{2A}- and α_{2C}-adrenoceptors expressed in CHO cells

Compound	pEC_{50} value[a]	
	α_{2A}-AR	α_{2C}-AR
Medetomidine	9.35 ± 0.28	9.02 ± 0.12
Methyl analog	8.79 ± 0.22	8.34 ± 0.24
(+)-Methyl isomer	9.09 ± 0.11[b]	8.65 ± 0.23
(−)-Methyl isomer	5.84 ± 0.10[b,c]	6.24 ± 0.22[c]
Desmethyl	8.31 ± 0.05[b]	8.96 ± 0.23
Trifluoromethyl	<5.00[d]	<5.00[e]

Data are expressed as the mean ± SEM of N = 4–5 experiments and data for the methyl analogs are plotted in Fig. 3.

[a] Values were determined as the negative log of effective concentration 50 (pEC_{50}) of each analog that reversed the effect of forskolin-induced cAMP response. Statistical analysis was carried out by one-way ANOVA and Student's *t* test at the 5% level of significance.

[b] Indicates that the pEC_{50} values for the analog on the α_{2A}-AR subtype are significantly different from the pEC_{50} values for the analog on the α_{2C}-AR subtype.

[c] Indicates that the pEC_{50} values for the (−)-isomer are significantly different from the pEC_{50} values for the racemate and (+)-methyl naphthyl imidazole on the α_{2A}- and α_{2C}-AR subtypes.

[d] Maximum percent inhibition at 10 μM = 24.

[e] Maximum percent inhibition at 10 μM = 25.

experiments to derive solution structures for each compound. As shown in Fig. 4, the ethylene in both compounds adopts a geometry orthogonal to the naphthyl ring projecting the methyl group into the plane of the naphthyl ring and away from the ring for the *Z* and *E* isomers, respectively. The most interesting aspect of these structures is the relative orientation of the imidazole ring wherein the C5H of the *E* isomer projects toward the naphthyl ring and the *Z* isomer any. The net effect of these orientations is to alter the positions of the potential hydrogen bond donor/acceptor pairs in the drug. This positioning of heteroatoms along with the orientations of the methyl groups may be responsible for forming favorable interaction of the *E*-ethylene with the α_{2C} relative to the other receptor types tested.

4. Discussion

Previous studies have subdivided the α_2-AR into α_{2A}-, α_{2B}- and α_{2C}-subtypes [14–16]. The aim has been to discover and develop subtype-selective agonists and antagonists; however, no major breakthrough has been made in this area [17]. The primary goal was to determine the importance of the methyl group of imidazole agonists and to establish whether the methyl pocket hypothesis could serve to identify novel α_2-AR subtype-selective agonists. In order to achieve this, the binding affinity of selected imidazole analogs was determined on human α-AR subtypes. The affinities of these analogs were compared at four human α-AR subtypes in competition with

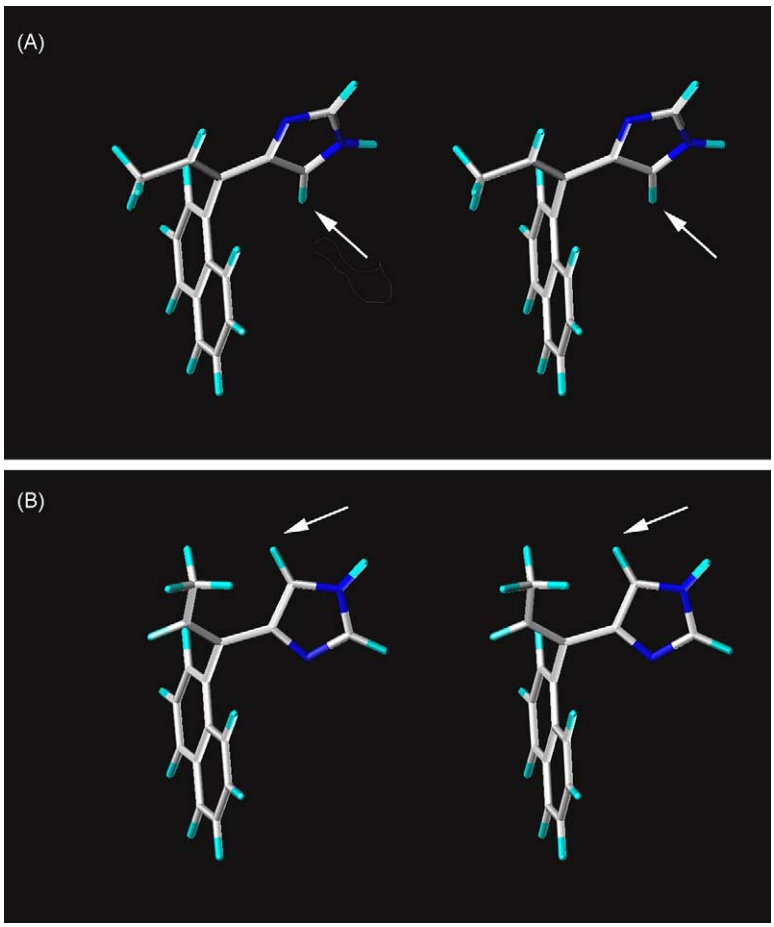

Fig. 4. Stereoscopic views of the *E*-ethylene isomer (panel A) and the *Z* isomer (panel B) showing the relative orientations of the side chains with respect to the naphthyl ring. The position of the C5H proton is shown by the arrow, carbon atoms are white, hydrogen atoms are cyan, and nitrogen atoms are blue.

[^{3}H]rauwolscine in CHO cells and [^{3}H]prazosin in HEK cells expressing these human subtypes. The functional potency of selected analogs were also determined on the human α_{2A}- and α_{2C}-AR subtype.

The results of our study with human α_{1A}-, α_{2A}-, α_{2B}- and α_{2C}-ARs demonstrate a high binding affinity for medetomidine, methyl naphthyl analog, and its optical isomers (Table 1) on the α_2-AR subtypes and an increased α_2/α_1-AR selectivity. This is in agreement with earlier studies using rat brain, wherein medetomidine, methyl naphthyl imidazole and its optical isomers demonstrated high receptor potencies and α_2/α_1-AR selectivity [7]. As compared to medetomidine and methyl naphthyl imidazole, the desmethyl naphthyl imidazole analog did not show an α_2- vs. α_1-AR selectivity in our current binding studies on the human subtypes, as has been reported in previous binding studies using rat brain [7].

Our results reveal that all of the naphthyl imidazole analogs showed lower binding affinities on the α_2-ARs as compared to naphthyl methyl analog and medetomidine. This information strongly indicates that the methyl group is important for ligand–receptor interactions on human α_2-ARs. The studies of binding with the stereoisomers of methyl naphthyl imidazole indicated that there were no changes in affinity, but that functionally, the *S*-(+)-methyl naphthyl imidazole was a much more potent agonist on α_{2A}- and α_{2C}-ARs than the *R*-(−)-isomer. The two rigid analogs, 2-naphthalene and 8-naphthalene were designed in a way that forces the side chain into different orientations, so that the dihedral angle of 8-naphthalene is close to that of methyl naphthyl imidazole and this could account for the high α_2-AR affinity of 8-naphthalene. However, the 8-naphthalene analog no longer retained α_2/α_1-AR selectivity, and implies that the conformational flexibility of the methyl group is important for optimal interaction with the α_2-ARs. Replacing the lipophilic methyl group of methyl naphthyl imidazole with polar substituents (hydroxy, methoxy or keto groups) caused a considerable reduction in α_2-AR affinity. Therefore, the lipophilicity of this group is important for binding to the α_2-AR. Steric size is also an important factor as replacing the methyl with ethyl or ethylene groups also decreased the α_2-AR affinity. Further, replacement of the methyl group with an electronegative trifluoromethyl group markedly reduced the affinity at the α_{1A}- and α_2-AR subtypes. This is interesting since the size of methyl and trifluoromethyl groups are nearly the same. Collectively, these findings suggest that the steric size, stereochemistry, electronic nature and liposolubility of the

substituent present at the benzylic carbon atom of naphthyl imidazole significantly influence the binding interactions with these α-AR subtypes.

Another major finding of our study is that we were able to obtain parallel differences in affinities among the three α_2-AR subtypes for analogs, *E*-ethylene, trifluoromethyl and hydroxy imidazole analogs, as compared to medetomidine. NMR and molecular modeling studies of *E*- and *Z*-ethylene isomers indicates that the imidazole ring in each compound adopts a unique geometry relative to the methyl group. This data suggests that the ligand binding pocket of the α_{2C}-AR contains a different juxtaposition of the methyl pocket and hydrogen bond donor/acceptor pairs relative to α_{2A}- and α_{2B}-AR. While it is possible that the ligand binds the receptor in an orientation different from that observed in solution, this observation does provide a new window into the design of novel ligands for the α_2-AR. In fact, we are now carrying out QSAR studies on these and other α-AR ligands to see if these observations can be exploited in our efforts to develop selective ligands.

Radioligand binding is only one method for probing for distinctions between receptor subtypes. Therefore, we have also described a transient transfection system using a 6 CRE-luciferase reporter gene plasmid in an attempt to correlate binding affinities of selected imidazole analogs with the functional changes in cAMP levels, an effect that is more downstream (post-receptor) and is a biochemical response of adenylyl cyclase that is initiated by ligand interactions with the α_2-AR. In the functional studies, medetomidine showed high functional potency for the human α_{2A}- and α_{2C}-AR subtypes ($EC_{50} = 0.45$ and 0.95 nM, respectively); and methyl naphthyl imidazole, and its desmethyl and trifluoromethyl analogs showed similar rank order in our functional studies as in binding studies.

Using the optical isomers of methyl naphthyl imidazole, we have also observed that chirality at the carbon bridge of methyl naphthyl imidazole is very important for agonist interactions with the α_{2A}- and α_{2C}-AR subtypes. Marked stereoselectivity [*S*-(+)-isomer ⩾ *R*-(−)-isomer] was noted in functional responses with the isomers of methyl naphthyl imidazole. This is in agreement with functional data obtained on α_2-ARs in guinea pig ileum, wherein *R*-(−)-methyl naphthyl imidazole showed no intrinsic activity and the *S*-(+)-methyl naphthyl imidazole showed enhanced α_2-AR agonist activity, as compared to the racemate [7]. The *S*-(+)-isomer of medetomidine was also more potent than the *R*-(−)-isomer on α-ARs. Thus, stereochemistry of the methyl group at the carbon bridge position of methyl naphthyl imidazole, like that of medetomidine, is important for the retention of agonist potency at the α_2-ARs.

The current studies with naphthyl imidazole analogs support the existence of the methyl pocket hypothesis. However, addition of the methyl group to the benzylic carbon position of 4-subsituted imidazoles such as medetomidine and its naphthalene analog does not increase the affinity on the α_2-AR subtypes, and decreases the affinity on the α_{1A}-AR subtype. Thus, it can be proposed that the α_2/α_1-AR selectivity, due to the addition of the methyl group could be an α_1-AR effect, such as a lack of space in the α_1-AR to accommodate the methyl group. Therefore, molecular modeling on the α_1-AR subtypes is necessary to confirm that the α_2-AR selectivity of medetomidine and methyl naphthyl imidazole is due to the interaction of the methyl group with the proposed pocket in the α_2-AR subtypes. Also molecular modeling studies are required to show that the imidazoline receptors do not possess the methyl pocket, which would ascertain that these compounds are selective for the α_2-ARs.

The results obtained in these studies are valuable since we have used the human receptors expressed in a homogenous system, instead of relying on the use of potential multiple populations of receptors from tissues of experimental animals. In addition, the CRE-luciferase assay may be used for studies on the functional, second messenger effects of the test compounds in parallel with the binding assays. Finally, the methyl pocket hypothesis along with the spatial orientations of functional groups could be used as an important tool for the development of α_2-AR subtype-selective ligands.

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