Structure−Activity Relationships of New 1*H*-Imidazo[4,5-*c*]quinolin-4-amine Derivatives as Allosteric Enhancers of the A_3 Adenosine Receptor

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1*H*-Imidazo[4,5-*c*]quinolin-4-amine derivatives have been synthesized as allosteric modulators of the human A_3 adenosine receptor (AR). Structural modifications were made at the 4-amino and 2 positions. The compounds were tested in both binding and functional assays, and many were found to be allosteric enhancers of the action of A_3AR agonists by several different criteria. First, a potentiation of the maximum efficacy of the agonist Cl-IB-MECA was observed for numerous derivatives. Also, a number of these compounds decreased the rate of dissociation of the agonist [^{125}I]I-AB-MECA from the A_3AR. Most prominently, compound **43** (LUF6000) was found to enhance agonist efficacy in a functional assay by 45% and decrease dissociation rate similarly without influencing agonist potency. The structural requirements for allosteric enhancement at the A_3AR were distinct from the requirements to inhibit equilibrium binding. Thus, we have prepared allosteric enhancers of the human A_3AR that have an improved allosteric effect in comparison to the inhibition of equilibrium binding at the orthosteric site.

Introduction

The adenosine receptors (ARs), a family of GPCRs consisting of the A_1, A_{2A}, A_{2B}, and A_3 subtypes, are activated by the ubiquitous modulator adenosine in our body. Selective agonists have been synthesized for three out of the four subtypes of adenosine receptors (ARs).[1−3] Each of these groups of subtype-selective agonists has representative examples that have already entered clinical trials.[1] A_1AR selective agonists are under development for treating cardiac arrhythmia, pain, and pulmonary disorders.[4−6] A_{2A}AR agonists are in clinical trials for cardiac stress imaging and are also of interest for inflammation.[7] A_3AR agonists, such as IB-MECA (*N*^{6}-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine, see Figure 1), are in clinical trials for colon carcinoma and rheumatoid arthritis and have potential for the treatment of myocardial infarction, stroke, and other disorders.[1]

Although newer and more selective AR agonists are being developed,[1,2] there are inherent problems, in general, associated with the therapeutic use of GPCR agonists.[8] The widespread occurrence of receptors such as the ARs makes selectivity for some organs or tissues nearly unachievable using directly acting (orthosteric) agonists, and thus a number of agonists were discontinued after initial phases of clinical trials.[1,2] In contrast to directly acting agonists, allosteric modulators act at a separate site on the receptor protein to modulate the effect of a native agonist.[9] An advantage of a positive allosteric modulator of a membrane receptor over its native, orthosteric activator is that greater selectivity, in principle, may be achieved. The positive allosteric modulator would enhance the action of the native agonist, but may have no effect of its own on the unoccupied receptor. Thus, the effect of endogenous agonist, which may be insufficient in a particular disease state, may be magnified in a temporally specific manner through allosteric modulation. The higher subtype-selectivity commonly exerted by allosteric modulators and the fact that the allosteric action is ideally coupled to simultaneous presence of the endogenous ligand both help to prevent over-dosage compared to the administration of a conventional, and possibly nonselective, orthosteric agonist.

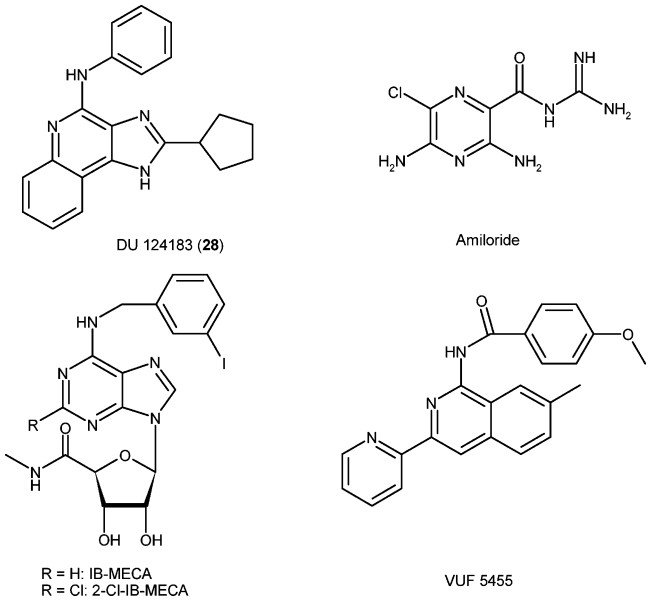

Figure 1. Structures of reference agonists (IB-MECA and 2-Cl-IB-MECA) and allosteric modulators (DU 124183, amiloride, and VUF5455) of the A_3AR.

Allosteric modulation is considered an important mechanism of controlling receptor function.[8,10−12] In the case of the ligand-gated ion channels, a series of benzodiazepines, including diazepam, enhance the CNS inhibitory function of the endog-

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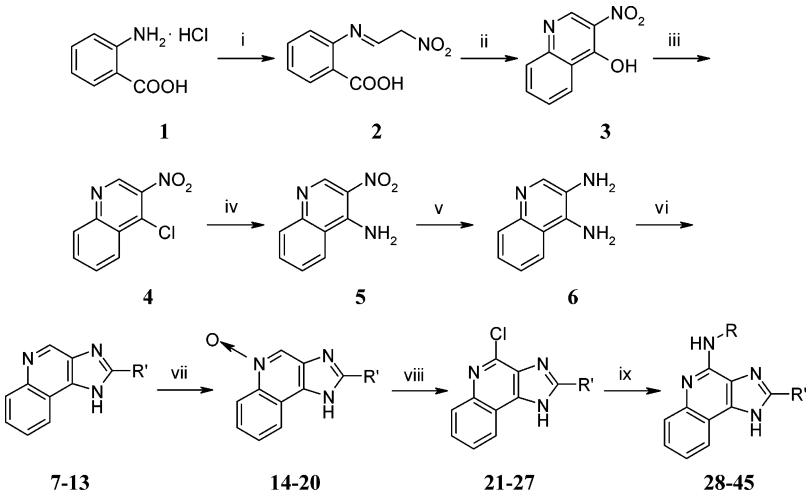
Scheme 1[a]



R' = H: **7**, **14**, **21**
R' = *c*-butyl: **8**, **15**, **22**
R' = *c*-pentyl: **9**, **16**, **23**
R' = *c*-hexyl: **10**, **17**, **24**
R' = *c*-heptyl: **11**, **18**, **25**
R' = 2-furyl: **12**, **19**, **26**
R' = pentyl: **13**, **20**, **27**

See Table 1

[a] Reagents: (i) HCl, HON=$CHCH_2NO_2$; (ii) $(CH_3CO)_2O$, CH_3COOK; (iii) $POCl_3$; (iv) NH_3; (v) H_2/Pd; (vi) (a) polyphosphoric acid, R′COOH, (b) trimethyl orthoformate, HCOOH, (c) 1. R′COCl, 2. NaOH; (vii) 3-chloroperoxybenzoic acid; (viii) $POCl_3$; (ix) RNH_2.

enous γ-aminobutyric acid, and have become the most widely prescribed sleep medications. In contrast, the directly acting agonists have not found clinical applications due to the inherent side effects. In the GPCR field, cinacalcet, a positive allosteric modulator of the calcium-sensing receptor (CaR), has recently been approved for the treatment of secondary hyperparathyroidism in dialysis patients suffering from chronic kidney disease.[13,14] In the case of ARs, the A_1AR has been the most studied, and one of its allosteric enhancers, T62 (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl-(4-chlorophenyl)methanone), is now in Phase I clinical trials for the potential treatment of neuropathic pain.[1,15]

Several classes of compounds (see also Figure 1) have previously been reported to be allosteric modulators for the A_3-AR, including the 3-(2-pyridinyl)isoquinoline (VUF5455),[16] the 1*H*-imidazo[4,5-*c*]quinolin-4-amine (DU124183, **28** in the current study),[17] and analogues of amiloride such as *N*,*N*-hexamethyleneamiloride.[18] One of the most interesting findings was that **28** and some of its analogues decreased agonist potency but enhanced maximum agonist efficacy.[17,19]

In the present study we have extended our earlier finding that 1*H*-imidazo[4,5-*c*]quinolin-4-amine derivatives act as allosteric modulators of the A_3AR.[17] We have synthesized and characterized the SAR (structure−activity relationship) of a new set of analogues based on compound **28**. A number of derivatives were found to be allosteric enhancers of the A_3AR. One of the analogues of **28**, compound **43**, was found to enhance agonist efficacy in a functional assay and decrease dissociation rate in binding but without influencing agonist potency.

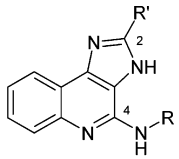
Results

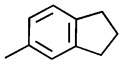
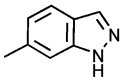
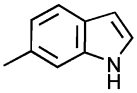
Chemistry. The novel derivatives **28**−**45** were synthesized as shown in Scheme 1. Condensation of anthranilic acid hydrochloride (**1**) with 2-nitroacetaldehyde oxime (HON=$CHCH_2NO_2$), prepared in situ from CH_3NO_2 and NaOH,[20] resulted in 2-(2-nitroethylideneamino)benzoic acid (**2**), which was dehydrated in acetic anhydride in the presence of potassium acetate to give 3-nitro-4-hydroxyquinoline (**3**).[20] 3-Nitro-4-hydroxyquinoline (**3**) was treated with phosphorus oxychloride to afford 3-nitro-4-chloroquinoline (**4**). This was converted to 3-nitro-4-aminoquinoline (**5**) with ammonia, which was subsequently reduced by catalytic hydrogenation to 3,4-diaminoquinoline (**6**) with 10% palladium on charcoal as catalyst. The next step involved ring-closure, which was carried out on three different ways. Compounds **8**−**11** were prepared by ring closure of the appropriate carboxylic acids and 3,4-diaminoquinoline (**6**) in polyphosphoric acid.[21] Compounds **12** and **13** were prepared by ring-closure of 2-furoyl chloride and hexanoyl chloride, respectively, with 3,4-diaminoquinoline (**6**).[22] Compound **7** was prepared by ring-closure of 3,4-diaminoquinoline (**6**) with formic acid in trimethylorthoformate. Oxidation with 3-chloroperoxybenzoic acid afforded 5-oxides **14**−**20**, which subsequently could be converted with phosphorus oxychloride into the respective 4-chloro compounds **21**−**27**. Finally, treatment of compounds **21**-**27** with the appropriate amines afforded the desired compounds **28**−**45**, respectively.[23]

Biological Activity. Previously reported compound **28** (ref 17) and newly synthesized imidazoquinolines **29**−**45** affect AR binding to or function at ARs as shown in Table 1. We first tested the effect of these compounds on the equilibrium binding at A_1, A_{2A}, and A_3ARs using standard agonist radioligands[16,17] [^{3}H]R-*N*^{6}-[phenylisopropyl]adenosine, [^{3}H]2-[*p*-(carboxyethyl)-phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine, and [^{125}I]-*N*^{6}-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide, respectively. We then measured the effect of these compounds (at 10 μM) on the A_{2B}AR with a cyclic AMP functional assay,[24] as an agonist radioligand has not been readily available for this subtype. Most compounds only induced a rather weak inhibition of equilibrium binding at the concentration used. For compounds **28**−**32** we also determined K_i values, if possible. It is not known whether the observed inhibition is of allosteric or nonallosteric character.

We further measured the capability of these compounds to influence the dissociation of an agonist radioligand, e.g. [^{125}I]I-AB-MECA at the A_3AR (Figure 2), which is a commonly used approach in the study of allosteric modulators[8,10] and perturbation of the dissociation rate reflects the potency of the allosteric modulators. It has been reported previously that compound **28** decreased the dissociation rate of [^{125}I]I-AB-MECA from the human A_3ARs.[17] Here it is shown that the dissociation rates of

Table 1. Potency of 1*H*-Imidazo[4,5-*c*]quinolin-4-amine Derivatives in Binding Assays at Human A_1, A_{2A}, A_{2B}, and A_3ARs Expressed in CHO Cells and Allosteric Effects at the Human A_3AR[a]



No.	*R*	*R'*	*K_i (hA_1AR), nM[a] or % displ. at 10 μM*	*K_i (hA_{2A}AR), nM[a] or % displ. at 10 μM*	*hA_{2B}AR % inhib. at 10 μM[b]*	*K_i (hA_3AR), nM[a] or % displ. at 10 μM*	*hA_3AR ag. Dissociation (%)[c] at 10 μM*	*Relative efficacy (%) at hA_3AR[d] at 10 μM*
28	Ph	CP	3420 ± 230	3150 ± 210	-6.8%	786 ± 67 (90%)	174±5	138±8
29	4-CH_3-Ph	CP	3850 ± 500	5220 ± 320	-4.1%	1190 ± 107 (87%)	153±4	128±4
30	4-CH_3O-Ph	CP	4170 ± 730	>10,000 (16%)	7.3%	410 ± 64 (92%)	166±11	132±4
31	3,4-Cl_2-Ph	CP	>10,000 (15%)	>10,000 (0%)	-6.7%	4690±970 (67%)	144±9	141±5
32	4-Cl-Ph	CP	>10,000 (22%)	>10,000 (17%)	-10.2%	1610 ± 550 (82%)	159±5	136±3
33	3-$HOCH_2$-Ph	CP	51%	49%	-4.1%	56%	129±15	118±4
34		CP	21%	8%	-10.7%	69%	108±4	111±5
35		CP	56%	68%	5.0%	67%	109±3	96±2
36	4-CH_3O-$PhCH_2$	CP	59%	60%	0.3%	80%	101±13	109±3
37		CP	70%	74%	10.1%	89%	126±9	125±2
38	$PhCH_2$	CP	28%	77%	-3.4%	86%	145±10	147±8
39	$Ph(CH_2)_2$	CP	52%	91%	-11.6%	84%	154±7	137±4
40	3,4-Cl_2-Ph	cycloheptyl	-4%	-2%	-7.3%	68%	130±2	115±7
41	3,4-Cl_2-Ph		-4%	70%	9.0%	78%	98±3	95±4
42	3,4-Cl_2-Ph	cyclobutyl	-5%	0.4%	-5.5%	52%	116±3	126±3
43	3,4-Cl_2-Ph (LUF6000)	cyclohexyl	-2%	-1%	-5.6%	45%	173±5	145±7
44	3,4-Cl_2-Ph	H	1.8%	-0.8%	10.2%	39%	91±7	92±4
45	3,4-Cl_2-Ph	*n*-pentyl	43.9%	-1%	-9.3%	84%	116±5	102±5

[a] All experiments were performed using adherent CHO cells stably transfected with cDNA encoding the human ARs. Binding at human A_1, A_{2A} and A_3ARs in this study was carried out as described in Experimental Procedures using [^{3}H]R-PIA, [^{3}H]CGS 21680, or [^{125}I]I-AB-MECA as a radioligand. Values from the present study are expressed as mean ± SEM, $n = 3-5$. Percentage inhibition at A_1, A_{2A}, or A_3 receptors is expressed as the mean value from two to four separate experiments with similar results performed in duplicate. [b] A_{2B}AR: effect of compounds at 10 μM on NECA (150 nM)-induced cyclic AMP accumulation from one experiment performed in triplicate, CGS15943 (10 μM) = 100%. [c] Dissociation: % decrease of [^{125}I]I-AB-MECA dissociation at 30 min (control = 100%). [d] Increase of efficacy: compared to maximal effect by 2-Cl-IB-MECA alone (control).

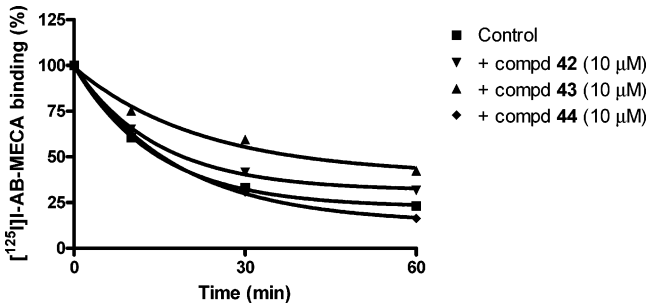


Figure 2. Radioligand binding studies on the human A_3AR. Study of the dissociation kinetics of the agonist radioligand [^{125}I]I-AB-MECA under control conditions and in the presence of 10 μM of compound **42**, **43**, or **44**.

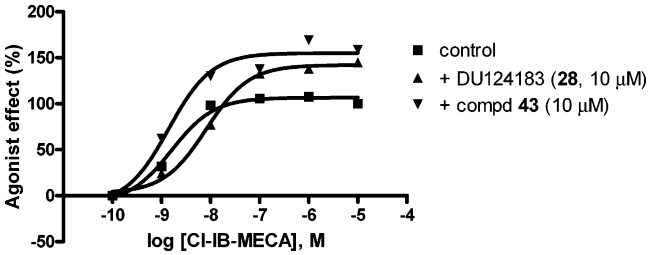


Figure 3. Functional assay of the human A_3AR. The % inhibition of forskolin-stimulated cAMP production by increasing concentrations of 2-Cl-IB-MECA under control conditions or in the presence of 10 μM of compound **28** or **43**.

[^{125}I]I-AB-MECA from the A_3AR in the presence of 10 μM **42** and **43** were 0.038 $\pm$ 0.004 and 0.036 $\pm$ 0.005 min^{-1}, respectively, which were significantly reduced and different from control (no further compound present) and that in the presence of 10 μM **44** (0.061 $\pm$ 0.006 min^{-1}). By comparing the ligand dissociation at 30 min in the absence or presence of 10 μM imidazoquinoline, a number of these newly synthesized compounds (compounds **28**−**33**, **37**−**40**, **43**) were found to decrease the dissociation significantly (Table 1). The ability of these compounds to affect the dissociation was found not to correlate with their ability to displace the equilibrium ligand binding at the A_3AR (Table 1, $r = 0.34$). For example, although **28** was more potent than **43** in displacing [^{125}I]I-AB-MECA binding, their ability to affect the dissociation rate was similar (Table 1).

We further examined the ability of these compounds to influence agonist function by using a cyclic AMP functional assay in intact CHO cells stably expressing the human A_3AR. The selective A_3 agonist Cl-IB-MECA concentration-dependently inhibited forskolin-stimulated cyclic AMP accumulation (Figure 3), corresponding to an EC_{50} of 3.8 $\pm$ 1.2 nM. Compound **28** concentration-dependently increased the maximum agonist efficacy but somewhat decreased agonist potency. Interestingly, one of the newly synthesized compounds (**43**) was found to increase agonist efficacy but did not influence agonist potency. The ability of compounds to influence agonist efficacy did not correlate with their ability to inhibit equilibrium ligand binding at the human A_3AR (Table 1, $r = 0.25$), further evidence of a complex mechanism of interaction. However, the ability of each compound to decrease the dissociation rate was positively correlated with its efficacy-enhancing effect (Table 1, $r = 0.88$).

Discussion

Allosteric modulation is considered of growing importance in controlling biological systems.[25] GPCRs are the single most important target in drug development. Thus, allosteric modulation of GPCRs is of particular importance.

The present study is actually an important extension of an earlier pharmacological study.[17] Previously it was found that functional enhancement of agonist action at the A_3AR by **28** (see Figure 1 and Table 1) and its analogues has potential for further development in the direction of therapeutic applications. A_3AR mutagenesis studies[18] have implicated amino acids F182$^{5.43}$ and N274$^{7.45}$ in the action of **28.** The mutagenesis data were interpreted using a rhodopsin-based A_3AR molecular model, suggesting multiple binding modes of the enhancers either at the orthosteric site or at a distinct putative allosteric site. Here we synthesized and systematically studied a new series of allosteric modulators for the A_3AR. Allosteric enhancers of A_3AR activation may be predicted to be useful against a number of disorders, including ischemic conditions and cancer.

It is interesting that compounds within this series showed a remarkable variety of allosteric modulatory effects, with positive, neutral, and maybe slightly negative effects on both agonist efficacy and ligand dissociation. It was shown that these compounds influenced agonist binding, potency, and efficacy in a separate manner. The identification of the important correlation between the ability of this series of compounds to affect ligand dissociation and their capability to enhance agonist efficacy suggested the possibility of further structural refinement to design more potent and efficacious allosteric enhancers.

Allosteric modulators that separately influence agonist affinity and efficacy at several GPCRs have been reported. For example, T62, which is now under clinical trails for potential treatment of neuropathic pain, has recently been suggested to enhance agonist efficacy rather than agonist potency as demonstrated in a functional assay of GTPγS binding to rat brain membranes.[15] CPCCOEt (7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate ethyl ester), a noncompetitive metabotropic glutamate receptor 1 antagonist, allosterically inhibits receptor signaling without affecting glutamate binding.[28] PIT (2,2′-pyridylisatogen tosylate) antagonizes $P2Y_1$ receptor signaling without inhibiting nucleotide binding.[29] Compound **28** enhanced agonist efficacy but decreased agonist potency (ref 17 and this study). In previous studies of modulation of various GPCRs by several series of compounds, allosteric potencies determined from dissociation kinetics have not been found to correlate with binding potency from equilibrium experiments.[19,26,27] However, to our knowledge, the findings from the current study represent the first evaluation of the correlation among the effects on agonist efficacy, dissociation kinetics, agonist potency, and affinity.

Agonist binding site(s) on a GPCR used to be conceived of as an orthosteric site in contrast to the site(s) at which allosteric modulators bind. In that framework, allosteric modulators are without effect in the absence of a ligand binding to the orthosteric site. However, recently, a number of compounds were found to be allosteric agonists. AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]piperidine) is an allosteric agonist for the M_1 muscarinic receptor.[30] Alcuronium has been shown to induce muscarinic receptor activation, which is not prevented by the classical muscarinic antagonist quinuclidinyl benzilate.[31] Thus, the activation mechanisms and structural and pharmacological modeling of GPCRs should also be reconstructed to accommodate the complex nature of GPCR activation. Allosteric modulation of GPCRs has recently been modeled in pharmacological terms,[32,33] to provide a theoretical basis for an allosteric modulator separately affecting affinity, potency, and efficacy. Hall[32] suggested that CPCCOEt is positively cooperative with the binding of glutamate but negatively cooperative with its

functional activation of the receptor. There is also evidence that some allosteric enhancers of agonist binding to the A_1AR have intrinsic activity themselves at that receptor.[9,34] However, the allosteric enhancers in the present study were not demonstrated to be allosteric agonists, as they did not show any effects by themselves.

Structure−activity relationships for the allosteric enhancing effects, based on slower dissociation of an A_3AR agonist radioligand, for the present series were evident. At the 4-amino position, a phenyl ring or substituted monocyclic phenyl ring was more enhancing than bicyclic aryl rings. Also, by the same criterion a phenyl group was more highly enhancing than the corresponding benzyl and phenylethyl groups. As noted previously[17] and consistent with compound **44**, substitution at the 2-position was necessary for allosteric enhancement. At this position, medium-sized cycloalkyl substituents (cyclopentyl and cyclohexyl) were most favorable for enhancement. Analogues bearing smaller or larger rings or an acyclic alkyl group were considerably less enhancing, and similarly an aromatic five-membered ring was not conducive to enhancement. With the criterion of increased maximal efficacy of a potent A_3AR agonist, benzyl and 3,4-dichlorophenyl groups at the 4-amino position were most enhancing.

In summary, by chemical structural modification we have surpassed the allosteric enhancement observed in the previous series of 1*H*-imidazo[4,5-*c*]quinolin-4-amine derivatives. Two compounds, 2-cyclopentyl-4-benzylamino (**38**) and 2-cyclohexyl-4-(3,4-dichlorophenyl)amino (**43**) analogues, potentiated the maximum efficacy of the agonist Cl-IB-MECA by 45−50%. Moreover, **43** enhanced agonist efficacy in a functional assay and decreased agonist dissociation rate without influencing agonist potency, probably because of its experimentally observed decreased interaction with the orthosteric binding site on the A_3AR (compare DU124128 (**28**) and **43**). In fact, most allosteric enhancers in the present study were generally weak in inhibition of ligand binding, an otherwise complicating factor in pharmacological evaluation. Thus, members of this series are useful as pharmacological probes for further studies of the A_3AR. Since the structural requirements for allosteric enhancement at the A_3AR are distinct from the requirements to inhibit equilibrium binding, there is hope of achieving even greater selectivity upon future structural manipulation.

Experimental Procedures

Instrumentation and Analysis. Microwave reactions were performed in an Emrys Optimizer (Biotage AB). Wattage was automatically adjusted so as to maintain the desired temperature. ^{1}H NMR spectra were measured at 200 MHz with a Bruker AC 200 or Bruker DMX 600 spectrometer. Chemical shifts for ^{1}H are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard, coupling constants are given in Hz. Melting points were determined with a Büchi capillary melting point apparatus and are uncorrected. Combustion analyses of new target compounds were performed by the analytical department of the Gorlaeus Laboratories, Leiden University (The Netherlands) and are within 0.4% of theoretical values unless otherwise specified.

Synthesis. 2-(2-Nitroethylideneamino)benzoic Acid (2). Prepared as described in the literature.[20] Yield: 18.40 g (89%): mp 196−197 °C.

3-Nitro-4-hydroxyquinoline (3). Prepared as described in the literature.[20] Yield: 6.93 g (49%): mp >300 °C.

3-Nitro-4-chloroquinoline (4). Prepared as described in the literature.[23] Yield: 5.05 g (81%): mp 118−119 °C.

3-Nitro-4-aminoquinoline (5). Prepared as described in the literature.[23] Yield: 6.1 g (95%): mp 255−257 °C.

3,4-Diaminoquinoline (6). Prepared as described in the literature.[23] Yield: 2.66 g (98%): mp 183−185 °C.

1*H*-Imidazo[4,5-*c*]quinoline (7). Prepared as described in the literature.[23] Yield: 0.44 g (81%): mp 263−265 °C.

General Procedure for 2-Substituted 1*H*-Imidazo[4,5-*c*]-quinolines (8−11). Polyphosphoric acid (1.3 mL/mmol) was added to 3,4-diaminoquinoline (**6**) and the appropriate carboxylic acid (1.2 equiv). The mixture was stirred at 100 °C for 5 h. Then it was cooled to 0 °C, and to it was added slowly NH_4OH till pH = 8−9. The mixture was extracted with ethyl acetate (3 × 15 mL), washed with water, brine, and again water, and dried over $MgSO_4$. The solution was filtered, the solvent was evaporated, and the residue was dried.

2-Cyclobutyl-1*H*-imidazo[4,5-*c*]quinoline (8). Scale: 6.2 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 1:1. Yield: 0.88 g (63%): mp 191−192 °C.

2-Cyclopentyl-1*H*-imidazo[4,5-*c*]quinoline (9). Scale: 4.0 mmol. Eluent for column chromatography was 5% methanol in dichloromethane. Yield: 0.77 g(81%): mp 191−192 °C.

2-Cyclohexyl-1*H*-imidazo[4,5-*c*]quinoline (10). Scale: 6.3 mmol. Eluent for column chromatography was 1−5% methanol in dichloromethane. Yield: 0.60 g (38%): mp 205−206 °C.

2-Cycloheptyl-1*H*-imidazo[4,5-*c*]quinoline (11). Scale: 6.3 mmol. Eluent for column chromatography was 1−5% methanol in dichloromethane. Yield: 0.45 g (27%): mp 225−226 °C.

2-(2-Furyl)-1*H*-imidazo[4,5-*c*]quinoline (12). 2-Furoyl chloride (1.1 g, 0.8 mL, 8.1 mmol) in dry dichloromethane (15 mL) was added dropwise to a solution of to 3,4-diaminoquinoline (1.0 g, 6.0 mmol) in dry pyridine (6.2 mL) under an atmosphere of nitrogen. The solution was stirred for 2 h at room temperature. Water (15 mL) was added to quench the reaction, and the solvent was evaporated under reduced pressure to afford an orange solid. This crude solid was refluxed for 2 h in 2 M NaOH (15 mL). After cooling on ice, the pH was adjusted to 7 using concentrated HCl. The solid was filtered off, washed with water and ether, extracted with ethyl acetate (3 × 15 mL), and washed with water (3 × 15 mL) again, and dried over $MgSO_4$. After evaporation, the residue was dried. Eluent for column chromatography was 1−5% methanol in dichloromethane. Yield: 0.62 g (44%): mp 236−238 °C.

2-Pentyl-1*H*-imidazo[4,5-*c*]quinoline (13). Prepared as described for the furyl compound (**12**) using hexanoyl chloride (1.75 g, 13 mmol). Eluent for column chromatography was ethyl acetate: petroleum ether 1:4 to 4:1. Yield: 0.85 g (41%): mp 142−143 °C.

General Procedure for 2-Substituted 1*H*-Imidazo[4,5-*c*]-quinolin-5-oxide (14−20). Starting material was almost completely dissolved (with heating) in chloroform (2.5 mL/mmol), dichloromethane (2.5 mL/mmol), and methanol (0.25 mL/mmol). 3-Chloroperoxybenzoic acid (2.5 equiv) was added, and the solution was refluxed. After 30 min Na_2CO_3 (0.04 g/mmol) was added, and the mixture was refluxed for an additional 1 h. The reaction mixture was cooled, and the solvent was evaporated. Column chromatography was needed for purification and removal of 3-chloroperoxybenzoic acid.

1*H*-Imidazo[4,5-*c*]quinolin-5-oxide (14). Scale: 8.3 mmol. Eluent for column chromatography was 2% methanol in dichloromethane. Yield: 0.55 g (36%): mp 290−295 °C.

2-Cyclobutyl-1*H*-imidazo[4,5-*c*]quinolin-5-oxide (15). Scale: 1.8 mmol. Eluent for column chromatography was 2−6% methanol in dichloromethane. Yield: 0.18 g (42%): mp 123−130 °C.

2-Cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-5-oxide (16). Scale: 0.8 mmol. Eluent for column chromatography was 5% methanol in dichloromethane. Yield: 0.19 g (95%): mp 155−157 °C.

2-Cyclohexyl-1*H*-imidazo[4,5-*c*]quinolin-5-oxide (17). Scale: 2.4 mmol. Eluent for column chromatography was 5−10% methanol in dichloromethane. Yield: 0.59 g (92%): mp 160−165 °C.

2-Cycloheptyl-1*H*-imidazo[4,5-*c*]quinolin-5-oxide (18). Scale: 2.0 mmol. Eluent for column chromatography was 3−8% methanol in dichloromethane. Yield: 0.22 g (39%): mp 115−120 °C.

2-(2-Furyl)-1*H*-imidazo[4,5-*c*]quinolin-5-oxide (19). Scale: 2.2 mmol. Eluent for column chromatography was 1−10% methanol in dichloromethane. Yield: 0.36 g (66%): mp >280 °C.

2-Pentyl-1*H*-imidazo[4,5-*c*]quinolin-5-oxide (20). Scale: 3.34 mmol. Eluent for column chromatography was 1−5% methanol in dichloromethane. Yield: 0.12 g (14%).

General Procedure for Substituted 4-Chloro-1*H*-imidazo[4,5-*c*]quinolines (21−27). A mixture of dry toluene (0.45 mL/mmol) and dry dimethylformamide (0.90 mL/mmol) was cooled in an ice bath, and phosphorus oxychloride (2.6 equiv) was added. After 10 min, the appropriate1*H*-imidazo[4,5-*c*]quinolin-5-oxide was added, and the solution was stirred at room temperature for 10 min. Subsequently the solution was heated to 100 °C for 30 min. Upon cooling, the solvent was evaporated, and the resulting syrup was poured on chipped ice while stirring. The mixture was then warmed to room temperature and carefully adjusted to pH 6−7 with solid $NaHCO_3$. After 2 h, the formed solid was filtered off, washed with water and di*iso*propyl ether, and subsequently dried.

4-Chloro-1*H*-imidazo[4,5-*c*]quinoline (21). Scale: 3.5 mmol. Eluent for column chromatography was 2% methanol in dichloromethane. Yield: 0.31 g (44%): mp 257−258 °C.

4-Chloro-2-cyclobutyl-1*H*-imidazo[4,5-*c*]quinoline (22). Scale: 0.6 mmol. Yield: 0.16 g (99%): mp 142−145 °C.

4-Chloro-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinoline (23). Scale: 12.7 mmol. Yield: 2.65 g (75%): mp > 265 °C.

4-Chloro-2-cyclohexyl-1*H*-imidazo[4,5-*c*]quinoline (24). Scale: 2.2 mmol. Yield: 0.65 g (97%): mp 245−250 °C.

4-Chloro-2-cycloheptyl-1*H*-imidazo[4,5-*c*]quinoline (25). Scale: 0.8 mmol. Yield: 0.38 g (86%): mp 195−200 °C.

4-Chloro-2-(2-furyl)-1*H*-imidazo[4,5-*c*]quinoline (26). Scale: 1.4 mmol. Eluent for column chromatography was ethyl acetate: petroleum ether = 25:75. Yield: 0.1 g (26%): mp 235−238 °C.

4-Chloro-2-pentyl-1*H*-imidazo[4,5-*c*]quinoline (27). Scale: 0.45 mmol. Yield: 0.052 g (41%): mp 236−237 °C.

General Procedure for *N*-Substituted 1*H*-Imidazo[4,5-*c*]-quinolin-4-amines (28−45). These compounds were prepared by means of microwave-assisted chemistry. Absolute ethanol (2.5−3.0 mL) was added to the appropriate 4-chloro-1*H*-imidazo[4,5-*c*]-quinoline and the appropriate aniline (2−3 equiv) under nitrogen. Conditions: prestirring 60 s, temperature 120 °C, time 2400 s, normal sample absorption, fixed hold time. After the reaction was completed, the solvent was evaporated and the remaining product was purified by column chromatography and recrystallized (MeOH/H_2O).

***N*-Phenyl-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (28).** Scale: 0.8 mmol. Eluent for column chromatography was 2.5−10% methanol in dichloromethane. Yield: 40 mg (15%): mp 155−157 °C.

***N*-(4-Methyl-phenyl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (29).** Scale: 0.7 mmol. Eluent for column chromatography was 1% methanol in dichloromethane. Yield: 80 mg (35%): mp 125−126 °C.

***N*-(4-Methoxy-phenyl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (30).** Scale: 0.7 mmol. Eluent for column chromatography was 1% methanol in dichloromethane. Yield: 90 mg (38%): mp 106−107 °C.

***N*-(3,4-Dichloro-phenyl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (31).** Scale: 0.7 mmol. Eluent for column chromatography was 0−2% methanol in dichloromethane. Yield: 150 mg (54%): mp 114−115 °C.

***N*-(4-Chloro-phenyl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (32).** Scale: 1.0 mmol. Eluent for column chromatography was 0.5−2% methanol in dichloromethane. Yield: 120 mg (33%): mp 189−190 °C.

***N*-(3-Hydroxymethyl-phenyl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (33).** Scale: 0.4 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 1:1, later increased to 7:3. Yield: 33 mg (25%): mp 228−230 °C.

***N*-([3,4-*c*]Indan-5-yl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (34).** Scale: 0.4 mmol. Eluent for column chromatography was 1% methanol in dichloromethane. Yield: 120 mg (81%): mp 142−145 °C.

***N*-(1*H*-Indazol-6-yl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (35).** Scale: 0.4 mmol. Eluent for column chromatography was 4% methanol in dichloromethane. Yield: 110 mg (74%): mp 235−236 °C.

***N*-(4-Methoxy-benzyl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (36).** Scale: 0.4 mmol. Eluent for column chromatography was di*iso*propyl ether. Yield: 33 mg (22%): mp 245−247 °C.

***N*-(1*H*-Indol-6-yl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (37).** Scale: 0.4 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 1:4, increased to 3:7. Yield: 30 mg (20%): mp 260−261 °C.

***N*-Benzyl-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (38).** Scale: 0.8 mmol. Eluent for column chromatography was 2% methanol in dichloromethane. Yield: 70 mg (25%), product was oily.

***N*-(Phenethyl)-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (39).** Scale: 0.8 mmol. Eluent for column chromatography was 1−4% methanol in dichloromethane. Yield: 160 mg (56%): mp 95−97 °C.

***N*-(3,4-Dichloro-phenyl)-2-cycloheptyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (40).** Scale: 0.8 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 1:9, increased to 100% ethyl acetate. Yield: 164 mg (50%): mp 236−238 °C.

***N*-(3,4-Dichloro-phenyl)-2-(2-furyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine (41).** Scale: 0.4 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 1:4. Yield: 96 mg (64%): mp 135−138 °C.

***N*-(3,4-Dichloro-phenyl)-2-cyclobutyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (42).** Scale: 0.7 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 1:4. Yield: 121 mg (48%): mp 131−134 °C.

***N*-(3,4-Dichloro-phenyl)-2-cyclohexyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (43).** Scale: 0.9 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 15:85, increased to 30:70. Yield: 160 mg (44%): mp 237−240 °C.

***N*-(3,4-Dichloro-phenyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine (44).** Scale: 1.0 mmol. Eluent for column chromatography was ethyl acetate:petroleum ether = 1:4. Yield: 160 mg (50%): mp 167−172 °C.

***N*-(3,4-Dichloro-phenyl)-2-pentyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (45).** Scale: 0.2 mmol. Eluent for column chromatography was dichloromethane. Yield: 44 mg (73%): mp 195−200 °C.

Pharmacological Methods. [^{125}I]*N*^{6}-(4-Amino-3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (I-AB-MECA; 2000 Ci/mmol), [^{3}H]*R*-PIA (R-*N*^{6}-[phenylisopropyl]adenosine, 34 Ci/mmol), [^{3}H]-CGS21680 (2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine, 47 Ci/mmol) and [^{3}H]cyclic AMP (40 Ci/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell Culture and Membrane Preparation. CHO (Chinese hamster ovary) cells expressing the recombinant human ARs (HEK-293 cells were used for the human A_{2A}AR) were cultured in DMEM and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. Cells were harvested by trypsinization. After homogenization and suspension, cells were centrifuged at 500*g* for 10 min, and the pellet was resuspended in 50 mM Tris·HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. The suspension was homogenized with an electric homogenizer for 10 s, and was then recentrifuged at 20 000*g* for 20 min at 4 °C. The resultant pellets were resuspended in buffer in the presence of 3 Units/mL adenosine deaminase, and the suspension was stored at −80 °C until the binding experiments. The protein concentration was measured using the Bradford assay.[35]

Binding Assays to the Human A_1 and A_{2A} ARs. For binding to the human A_1 AR, [^{3}H]R-PIA (2 nM) was incubated with membranes (40 μg/tube) from CHO cells stably expressing the human A_1 AR at 25 °C for 60 min in 50 mM Tris·HCl buffer (pH 7.4; $MgCl_2$, 10 mM) in a total assay volume of 200 μL. Nonspecific

binding was determined using 10 μM of N^6-cyclopentyladenosine. For human A_{2A} AR binding, membranes (20 μg/tube) from HEK-293 cells stably expressing the human A_{2A} AR were incubated with 15 nM [^{3}H]CGS21680 at 25 °C for 60 min in 200 μL of 50 mM Tris.HCl, pH 7.4, containing 10 mM $MgCl_2$. *N*-5′-Ethyluronamidoadenosine (10 μM) was used to define nonspecific binding. Reaction was terminated by filtration with GF/B filters.

Binding Assay to the Human A_3 AR. Each tube in the competitive equilibrium binding assay contained 100 μL of membrane suspension (20 μg protein), 50 μL of [^{125}I]I-AB-MECA (0.5 nM), and 50 μL of increasing concentrations of the test ligands in Tris·HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM of 5′-*N*-ethylcarboxamidoadenosine in the buffer. The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburg, MD). Filters were washed three times with 9 mL of ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter.

Dissociation Kinetics of [^{125}I]I-AB-MECA from Human A_3ARs. The dissociation of [^{125}I]I-AB-MECA was measured as follows. Membranes (20 μg) were preincubated at 25 °C with 0.5 nM [^{125}I]I-AB-MECA, in a total volume of 100 μL of Tris·HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, and 1 mM EDTA for 60 min. The dissociation was then initiated by the addition of 3 μM Cl-IB-MECA with or without allosteric modulators. The time course of dissociation of total binding was measured by rapid filtration at appropriate time intervals. Nonspecific binding was measured after 60-min incubation in the presence of 3 μM Cl-IB-MECA. Binding reactions were terminated as described above.

Cyclic AMP Accumulation Assay. Intracellular cyclic AMP levels were measured with a competitive protein binding method.[24] CHO cells that expressed recombinant human A_3ARs were harvested by trypsinization. After centrifugation and resuspension in medium, cells were plated in 24-well plates in 0.5 mL of medium. After 24 h, the medium was removed and cells were washed three times with 1 mL of DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with agonists and/or test compounds in the presence of rolipram (10 μM) and adenosine deaminase (3 units/mL). After 45 min, forskolin (10 μM) was added to the medium, and incubation was continued an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL of 0.1 M HCl or 50 μL of cyclic AMP solution (0−16 pmol/200 μL for standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters and washed once with cold buffer. Bound radioactivity was measured by liquid scintillation spectrometry.

Statistical Analysis. Binding and functional parameters were calculated using Prism 5.0 software (GraphPAD, San Diego, CA). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng−Prusoff equation.[36] Data were expressed as mean ± standard error. The Pearson correlation coefficients (r) between efficacy, binding, and dissociation were calculated using SYSTAT 11 (SYSTAT Software Inc.). The Pearson correlation coefficient is between −1 and 1, with 1 meaning that two series are positively correlated, 0 meaning that they are completely uncorrelated, and −1 meaning they are perfectly negatively correlated.

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Supporting Information Available: Tables with ^{1}H and ^{13}C NMR spectroscopic data and elemental analyses are available for selected compounds including all target compounds (**28**−**45**). This material is available free of charge via the Internet at http://pubs.acs.org.

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