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## A Series of 2,4-Disubstituted Quinolines as a New Class of Allosteric Enhancers of the Adenosine A<sub>3</sub> Receptor

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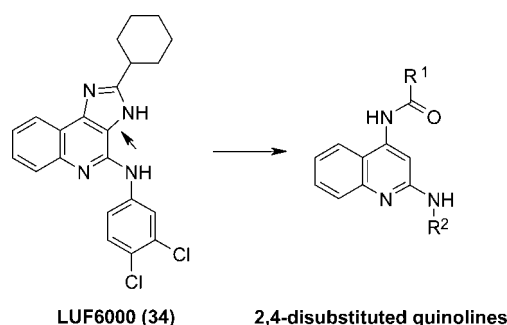
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The adenosine receptor subfamily consists of the adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors, which are localized in a variety of tissues throughout the human body. It is, therefore, a challenge to develop receptor specific ligands with improved tissue selectivity. Allosteric modulators could have these therapeutic advantages over orthosteric ligands. In the present study, a series of 2,4-disubstituted quinolines were synthesized on the basis of the structure of LUF6000 (**34**). Compound **27** (LUF6096) was able to allosterically enhance agonist binding to a similar extent as **34**. In addition, this new compound showed low, if any, orthosteric affinity for any of the adenosine receptors. In a functional assay, compound **27** showed improved activity in comparison to **34**, as it increased both the intrinsic efficacy and the potency of the reference agonist Cl-IB-MECA at the human adenosine A<sub>3</sub> receptor.

### Introduction

Adenosine receptors belong to the class A family of membrane-bound G-protein-coupled receptors (GPCRs).<sup>1</sup> The endogenous ligand adenosine and its receptors play an important physiological role, and together they mediate a large variety of effects, for example, on the cardiovascular, immune, and central nervous systems.<sup>2</sup> The adenosine receptor family consists of four subtypes, namely, the adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors, which are localized throughout the human body.

Several compound classes, both agonists and antagonists, have been described as (orthosteric) ligands for the adenosine receptors.<sup>3–6</sup> Over the years, medicinal chemists succeeded in the development of subtype selective ligands, with the possible exception of agonists for the adenosine A<sub>2B</sub> receptor.<sup>5</sup> However, many organs and tissues in the body express adenosine receptors, which has compromised the development of clinical candidates that have true in vivo selectivity of action. Allosteric modulation of a receptor target may have the therapeutic advantage of greater receptor subtype selectivity and tissue specificity.<sup>7</sup> In the past 2 decades, it has been observed that several GPCRs from different classes can be allosterically modulated, such as muscarinic receptors (class A), the corticotropin-releasing factor<sub>1</sub> (class B), and glutamate (class C) receptors.<sup>8,9</sup> Both allosteric



**Figure 1.** Structure of the reference allosteric enhancer (**34**) and the general structure of the new allosteric enhancers (2,4-disubstituted quinolines) of the adenosine A<sub>3</sub> receptor. The arrow points to the bond deletion that led to the new series.

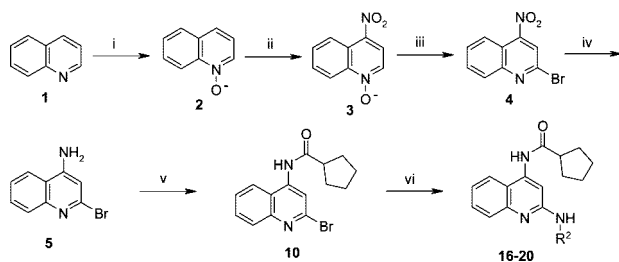
enhancers and inhibitors have been described that can either enhance or inhibit the affinity and/or potency of the endogenous ligand.<sup>8</sup> A similar situation has emerged for adenosine receptors. Nonselective modulators such as sodium ions and amiloride derivatives influence orthosteric ligand binding to adenosine A<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub> receptors.<sup>10,11</sup> More selective allosteric modulators also exist, such as PD81,723, an allosteric enhancer of agonist binding at the adenosine A<sub>1</sub> receptor.<sup>12</sup> On human adenosine A<sub>3</sub> receptors, 3-(2-pyridinyl)isoquinoline (VUF5455)<sup>13</sup> and 1*H*-imidazo[4,5-*c*]quinolin-4-amine (DU124183) were shown to be allosteric enhancers.<sup>14</sup> However, these compounds also inhibited equilibrium binding at the orthosteric site of the receptor. Recently, in a series of imidazoquinolinamines a novel allosteric enhancer of this receptor, (2-cyclohexyl-3*H*-imidazo[4,5-*c*]quinolin-4-yl)-(3,4-dichlorophenyl)amine (**34**), stood out because it had improved allosteric over orthosteric properties.<sup>15</sup>

Our aim for the present study was to find a new chemical template as the basis for novel allosteric modulators of the adenosine A<sub>3</sub> receptor with negligible, if any, orthosteric effects, and selectivity over the A<sub>1</sub> receptor. Therefore, the imidazoquinoline heterocyclic ring system of reference compound **34** was opened at the position indicated with an arrow (Figure 1), which resulted in a new compound class, namely, the 2,4-disubstituted quinolines. A series of these compounds with

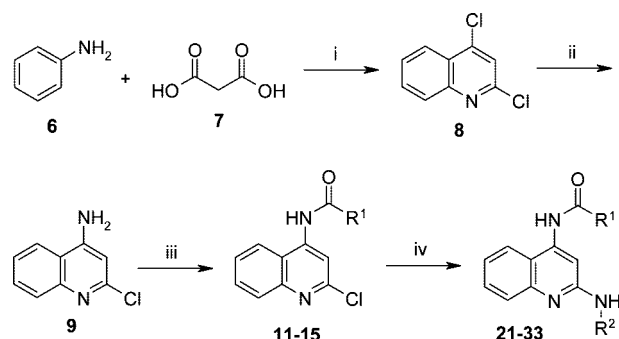
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<sup>†</sup> Both authors contributed equally to this study.

<sup>a</sup> Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosine 5'-monophosphate; CGS 21680, 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine; CHAPS, 3-[(3- cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; Cl-IB-MECA, 2-chloro-*N*-(3-iodobenzyl)-5'-*N*-methylcarbamoyladenosine; DCM, dichloromethane; DMSO, dimethyl sulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein-coupled receptor; I-AB-MECA, *N*-(4-amino-3-iodobenzyl)-5'-*N*-methylcarbamoyladenosine; LUF6000, (2-cyclohexyl-3*H*-imidazo[4,5-*c*]quinolin-4-yl)-(3,4-dichlorophenyl)amine; MRS1754, *N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)-phenoxy]acetamide; NECA, adenosine-5'-*N*-ethylcarboxamide; PBS, phosphate-buffered saline; R-PIA, *R*-phenylisopropyl; SAR, structure-activity relationship; SEM, standard error of the mean; TR-FRET, time-resolved fluorescence resonance energy transfer; ZM241385, 4-(2-[7-amino-2-(2-furyl){1,2,4}triazolo{2,3-*a*}{1,3,5,}-triazin-5-yl amino]ethyl)phenol.

Scheme 1<sup>a</sup>

<sup>a</sup> Reagents and conditions: (i)  $\text{CH}_3\text{COOH}$ ,  $\text{H}_2\text{O}_2$ , 70 °C; (ii)  $\text{H}_2\text{SO}_4$ ,  $\text{HNO}_3$ , 65 °C; (iii)  $\text{POBr}_3$ , 0 °C; (iv)  $\text{CH}_3\text{COOH}$ , Fe, 65 °C; (v) pyridine, acid chloride, 115 °C; (vi) amines, EtOH (140 °C, 80 min) or without solvent (180 °C, 90 min) in microwave.

Scheme 2<sup>a</sup>

<sup>a</sup> Reagents and conditions: (i)  $\text{POCl}_3$ , reflux; (ii)  $\text{NH}_4\text{OH}$ , 160 °C, 2.5 h in microwave; (iii) pyridine, acid chloride, 60 °C; (iv) amines, EtOH (140 °C, 80 min) or without solvent (180 °C, 90 min) in microwave.

similar substituents as described by Göblyös et al. were synthesized, and their structure–activity relationship (SAR) was examined.<sup>15</sup> The results obtained from radioligand displacement and kinetic dissociation experiments as well as functional (cAMP-based) assays were compared with the effects of **34**.

## Results and Discussion

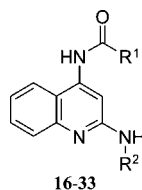
**Chemistry.** The novel derivatives **16–20** were synthesized as shown in Scheme 1. Oxidation of quinoline resulted in quinoline 1-oxide (**2**),<sup>16,17</sup> which was nitrated to give 4-nitroquinoline 1-oxide (**3**).<sup>18</sup> 4-Nitroquinoline 1-oxide (**3**) was treated with phosphorus oxybromide to afford 2-bromo-4-nitroquinoline (**4**).<sup>19,20</sup> This was converted into 4-amino-2-bromoquinoline (**5**) with iron powder in acetic acid.<sup>21</sup> Reaction of compound **5** with carbonyl chlorides<sup>22</sup> and then subsequently with the appropriate amines afforded the desired compounds **16–20**.<sup>15</sup> Derivatives **21–33** were obtained as depicted in Scheme 2. Ring-closure reaction of malonic acid with aniline in phosphorus oxychloride gave 2,4-dichloroquinoline (**8**),<sup>23</sup> which was subsequently treated with ammonia under microwave conditions to give 2-amino-4-chloroquinoline (**9**).<sup>24,25</sup> Reaction of compound **9** with carbonyl chlorides<sup>22</sup> and then subsequently with the appropriate amines afforded the desired compounds **21–33**.<sup>15</sup>

**Structure–Activity Relationships.** All newly synthesized compounds (**16–33**) and the reference compound (**34**, Figure 1) were tested in equilibrium and kinetic radioligand binding assays (Table 1). At first, equilibrium displacement assays were performed with <sup>125</sup>I-AB-MECA on CHO cells expressing the human adenosine A<sub>1</sub> or A<sub>3</sub> receptor. In addition, their effect on [<sup>3</sup>H]ZM241385 and [<sup>3</sup>H]MRS1754 equilibrium binding at the adenosine A<sub>2A</sub> and A<sub>2B</sub> receptors was examined. From Table 1 it follows that compounds **16–33** only moderately inhibited radioligand binding to the adenosine A<sub>1</sub> or A<sub>3</sub> receptor at 10

μM. For example, compounds **22** and **23** showed the highest inhibition of equilibrium radioligand binding at the adenosine A<sub>1</sub> and A<sub>3</sub> receptor, respectively. Notably, compound **30** inhibited radioligand binding at both the adenosine A<sub>2A</sub> and A<sub>2B</sub> receptor. In addition, compound **17** seemed to increase radioligand binding at the adenosine A<sub>2B</sub> receptor; however, the same effect was observed on membranes of CHO cells without the A<sub>2B</sub> receptor expressed (data not shown). The results obtained for the reference modulator **34** in this study were comparable to previously reported data,<sup>15</sup> although some inhibition of binding at the adenosine A<sub>1</sub> receptor was observed in the present study (39% vs –2%, respectively).

As compounds **16–33** were synthesized as possible allosteric modulators, their effect on the dissociation rate of <sup>125</sup>I-AB-MECA was investigated, as a measure for allosterism (Table 1).<sup>15</sup> It was previously reported that **34** decreased the dissociation rate of <sup>125</sup>I-AB-MECA from human adenosine A<sub>3</sub> receptors.<sup>7</sup> This was confirmed in the present study, as the amount of radioligand bound to the receptor was increased in comparison to the control condition (Table 1). Compound **34** did not influence the dissociation rate of <sup>125</sup>I-AB-MECA from the adenosine A<sub>1</sub> receptor, while equilibrium binding was inhibited to some extent on both adenosine A<sub>1</sub> and A<sub>3</sub> receptors. The latter strengthens the hypothesis that the allosteric site, and not the orthosteric site, is not evolutionarily constrained, which may result in a higher selectivity within a receptor subfamily.<sup>15</sup> Several compounds with a cyclopentyl substituent (**16–23**) at the R<sup>1</sup> position decreased the dissociation rate of <sup>125</sup>I-AB-MECA from the adenosine A<sub>3</sub> receptor, while the dissociation rate from the adenosine A<sub>1</sub> receptor was not influenced (Table 1). Typically, combination with a (substituted) phenyl ring on the R<sup>2</sup> position resulted in the most potent allosteric enhancers. However, introduction of a benzyl (**18**), cyclopentyl (**22**), or indazole (**23**) group on the R<sup>2</sup> position abolished the effect on the dissociation rate from the adenosine A<sub>3</sub> receptor. The most potent allosteric enhancer with a cyclopentyl substituent at R<sup>1</sup> was obtained in combination with a 3,4-dichlorophenyl group at R<sup>2</sup> (**17**). Subsequently, some analogues were synthesized with a smaller cyclobutyl (**24** and **25**) and a larger cyclohexyl (**26–29**) moiety at the R<sup>1</sup> position. Introduction of cyclobutyl instead of cyclopentyl resulted in compounds with lower modulating potency for a phenyl (**24** vs **16**) or a 3,4-dichlorophenyl (**25** vs **17**) substituent at R<sup>2</sup>. In combination with a cyclohexyl at R<sup>1</sup>, however, these substituents resulted in a strongly increased potency, **26** and **27**, respectively. A 4-methylphenyl (**28**) and an indane (**29**) substituent also resulted in highly potent allosteric enhancers. In general, the cyclohexyl group at R<sup>1</sup> yielded compounds with a higher potency than with a cyclopentyl one. Notably, compound **27** was the most potent enhancer of these series and showed the highest structural similarity to **34**. Moreover, compound **27** showed a decreased orthosteric effect on the adenosine A<sub>1</sub> and A<sub>3</sub> receptors when compared to **34** (Table 1). In addition, analogues were synthesized with a furan (**30** and **31**) or a phenyl ring (**32** and **33**). Although these rings have a similar size as the cyclopentyl (**16** and **17**) or cyclohexyl ring (**26** and **27**), respectively, the potency of the corresponding compounds was completely abolished. Apparently, an aromatic instead of an aliphatic substituent at the R<sup>1</sup> position is not tolerated.

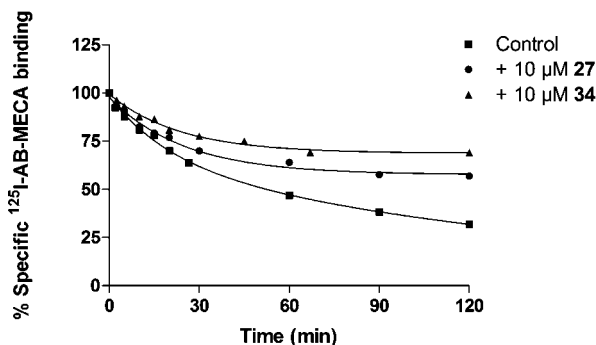
In the present study, compound **27** was the most potent allosteric enhancer of the adenosine A<sub>3</sub> receptor. Therefore, the effect of **27** on the dissociation rate of <sup>125</sup>I-AB-MECA from the A<sub>3</sub> receptor was further investigated in comparison to **34** (Figure 2 and Table 2). Dissociation was induced by the addition

**Table 1.** Affinity and Modulatory Potency of Compounds **16–33** and That of the Reference Allosteric Enhancer **34** at the Human Adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> Receptor Subtypes, Expressed as % Displacement and % Enhancement at 10 μM, Respectively<sup>d</sup>

No.	R <sup>1</sup>	R <sup>2</sup>	% Displacement <sup>a</sup>				% Enhancement <sup>b</sup>	
			hA <sub>1</sub>	hA <sub>2A</sub>	hA <sub>2B</sub>	hA <sub>3</sub>	hA <sub>1</sub>	hA <sub>3</sub>
16	cpentyl	phenyl	49	33	34	61	104	145
			(44/55)	(29/36)	(33/34)	(56/66)	(93/115)	(142/147)
17	cpentyl	3,4-diCl-phenyl	17	26	-74	50	125	169
			(17/17)	(21/31)	(-69/-79)	(45/55)	(115/134)	(168/170)
18	cpentyl	benzyl	34	34	49	28	77	107
			(30/37)	(33/34)	(45/52)	(23/34)	(66/88)	(105/108)
19	cpentyl	4-Me-phenyl	52	27	35	63	115	165
			(47/58)	(24/29)	(33/37)	(61/64)	(100/130)	(160/170)
20	cpentyl		47	36	14	62	90	147
			(42/52)	(35/36)	(13/14)	(60/64)	(90/90)	(143/150)
21	cpentyl	4-OMe-phenyl	24	9	19	35	94	134
			(22/25)	(7/10)	(15/22)	(24/45)	(74/114)	(131/137)
22	cpentyl	cpentyl	80	42	72	14	107	114
			(80/80)	(36/48)	(65/79)	(9/19)	(98/116)	(113/114)
23	cpentyl		61	52	56	90	72	102
			(55/66)	(51/53)	(47/74)	(88/91)	(61/83)	(101/102)
24	cbutyl	phenyl	76	73	79	66	79	119
			(67/85)	(72/74)	(76/82)	(64/69)	(74/83)	(116/122)
25	cbutyl	3,4-diCl-phenyl	22	41	38	51	72	149
			(18/26)	(40/42)	(37/38)	(49/52)	(61/83)	(149/149)
26	chexyl	phenyl	17	21	5	26	70	210
			(13/22)	(20/21)	(-2/11)	(24/28)	(68/72)	(205/214)
27	chexyl	3,4-diCl-phenyl	12	12	-19	17	94	249
			(9/15)	(12/12)	(-17/-20)	(16/17)	(73/114)	(264/234)
28	chexyl	4-Me-phenyl	63	-3	-3	64	121	220
			(62/65)	(-6/1)	(-1/-4)	(63/65)	(120/122)	(228/211)
29	chexyl		20	10	19	42	118	217
			(13/26)	(4/15)	(18/19)	(37/46)	(111/124)	(213/221)
30		phenyl	73	92	95	60	68	100
			(70/75)	(91/92)	(92/97)	(60/60)	(55/81)	(98/101)
31		3,4-diCl-phenyl	11	15	10	13	69	96
			(8/14)	(12/18)	(9/10)	(7/18)	(59/79)	(93/99)
32	phenyl	phenyl	64	46	75	76	77	100
			(61/66)	(43/49)	(69/80)	(76/76)	(70/83)	(98/101)
33	phenyl	3,4-diCl-phenyl	23	11	-20	37	102	112
			(18/27)	(10/11)	(-17/-22)	(36/38)	(101/103)	(106/117)
34 <sup>c</sup>			39	3	16	41	120	229
			(39/39)	(0/5)	(13/18)	(38/44)	(132/107)	(222/236)

<sup>a</sup> Displacement of specific <sup>125</sup>I-AB-MECA (A<sub>1</sub> and A<sub>3</sub>), [<sup>3</sup>H]ZM241385 (A<sub>2A</sub>), or [<sup>3</sup>H]MRS1754 (A<sub>2B</sub>) binding at their respective cell membranes. <sup>b</sup> Dissociation of <sup>125</sup>I-AB-MECA induced by an excess DPCPX (A<sub>1</sub>) or CI-IB-MECA (A<sub>3</sub>) in the absence (control) and presence of modulator. Enhancement of radioligand binding was determined after 90 (A<sub>1</sub>) or 120 min (A<sub>3</sub>) of dissociation, where the control was set at 100%. Values are the mean of two separate assays performed in duplicate. <sup>c</sup> See Figure 1 for the chemical structure of **34**. <sup>d</sup> All experiments were performed using CHO cells stably transfected with the human adenosine A<sub>1</sub>, A<sub>2B</sub>, or A<sub>3</sub> receptor or HEK293 cells stably transfected with the human adenosine A<sub>2A</sub> receptor.



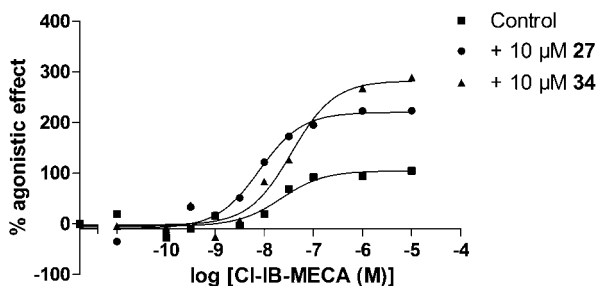


**Figure 2.** Dissociation kinetics of  $^{125}\text{I}$ -AB-MECA binding to human adenosine  $A_3$  receptors stably expressed in CHO cell membranes. Dissociation was initiated by either the addition of  $10\ \mu\text{M}$  CI-IB-MECA mixed with either buffer (control) or modulator. Shown are representative results from one experiment performed in duplicate (see Table 2 for kinetic parameters).

**Table 2.** Influence of **27** and **34** on the Dissociation Kinetics of the Radiolabeled Agonist  $^{125}\text{I}$ -AB-MECA from the Human Adenosine  $A_3$  Receptor Expressed on Membranes of Stably Transfected CHO Cells

condition	dissociation assay <sup>a</sup>	
	$k_{\text{off}}$ ( $\text{min}^{-1}$ )	shift $k_{\text{off}}$
control	$0.089 \pm 0.043$ (fast)	
	$0.0072 \pm 0.0011$ (slow)	
+10 $\mu\text{M}$ <b>27</b>	$0.029 \pm 0.014$	3.1
+10 $\mu\text{M}$ <b>34</b>	$0.035 \pm 0.008$	2.5

<sup>a</sup> The value of the kinetic dissociation rate constant was obtained by analysis of the exponential dissociation curve of  $^{125}\text{I}$ -AB-MECA bound to human adenosine  $A_3$  receptors in the absence (control) or presence of modulator. The shift is defined as the ratio of  $k_{\text{off}}$  values in the presence and absence (control, fast component) of modulator, respectively. Values are the mean ( $\pm$ SEM) of three separate assays performed in duplicate.



**Figure 3.** Modulatory effects of **27** and **34** on the effect–concentration curve of CI-IB-MECA for the inhibition of the forskolin-stimulated cAMP production in CHO cells expressing the human adenosine  $A_3$  receptor. Representative graphs from one experiment performed in duplicate (see Table 3 for  $\text{EC}_{50}$  and  $E_{\text{max}}$  values).

of an excess of CI-IB-MECA, which resulted in dissociation rates of  $0.089 \pm 0.043$  and  $0.0072 \pm 0.0011\ \text{min}^{-1}$  for a fast and slow phase, respectively. In the presence of **34** the dissociation rate decreased by 3.1-fold ( $k_{\text{off}} = 0.029\ \text{min}^{-1}$ ) when compared to the fast phase of dissociation in the absence of modulator, which corresponds to the previously reported effect of the same compound.<sup>15</sup> In a similar fashion, the dissociation rate was decreased 2.5 times ( $k_{\text{off}} = 0.035\ \text{min}^{-1}$ ) in the presence of **27**.

The effects of **27** and **34** were also investigated in a functional assay by measuring the inhibition of cAMP production by the reference agonist CI-IB-MECA (Figure 3 and Table 3). Both compounds ( $10\ \mu\text{M}$ ) significantly and dramatically enhanced the intrinsic activity of CI-IB-MECA to 223% for **27** and 286% for **34**. A similar study for **34** had been performed previously, in the presence of  $10\ \mu\text{M}$  rather than  $1\ \mu\text{M}$  forskolin as in the

**Table 3.** Inhibitory Effect (Potency and Intrinsic Activity) of CI-IB-MECA on cAMP Production in CHO Cells Expressing the Human Adenosine  $A_3$  Receptor in the Absence and Presence of Either **27** or **34** ( $10\ \mu\text{M}$ )

compd	activity in cAMP Assay <sup>a</sup>	
	$\text{EC}_{50}$ (nM)	$E_{\text{max}}$ (%)
CI-IB-MECA	$31 \pm 3$	$100 \pm 1$
+10 $\mu\text{M}$ <b>27</b>	$9 \pm 1^{***}$	$223 \pm 10^{***}$
+10 $\mu\text{M}$ <b>34</b>	$22 \pm 4$	$286 \pm 39^{**}$

<sup>a</sup> Inhibition of forskolin-induced cAMP production in CHO cells that stably express the human adenosine  $A_3$  receptors by CI-IB-MECA in the absence (control) and presence of modulator, with 100% as the amount of cAMP production in the presence of  $10\ \mu\text{M}$  CI-IB-MECA and 0% as the amount of forskolin-stimulated cAMP production. Values are the mean ( $\pm$ SEM) of three separate assays performed in duplicate: (\*\*)  $p < 0.005$ ; (\*\*\*)  $p < 0.001$  versus control.

present study. Under those conditions **34** was also capable of enhancing the intrinsic activity of CI-IB-MECA but now to approximately 150%.<sup>15</sup> Interestingly, in the present study **27** also enhanced the potency (expressed as  $\text{EC}_{50}$  value) of CI-IB-MECA, from 31 to 9 nM, whereas **34** did not, the latter in line with previously reported findings.<sup>15</sup> Notably, **27** and **34** do not display intrinsic efficacy at the human adenosine  $A_3$  receptor when applied alone (see point along y-axis, Figure 3) and can, therefore, be classified as true allosteric enhancers.<sup>26</sup> Similarly, two classes of allosteric enhancers have been reported for the GABA<sub>B</sub> receptor that increase agonist potency and efficacy without having intrinsic efficacy.<sup>27,28</sup> Urwyler and co-workers postulated that these compounds will have a better side effect profile because a synergistic effect will only arise in the presence of the endogenous agonist. As the adenosine receptor is expressed throughout the body, an allosteric enhancer such as compound **27** may therefore be more beneficial than an (allosteric) ligand that has intrinsic efficacy on its own.

## Conclusions

In summary, it was shown that scission of a chemical bond in the structure of the reference compound **34** led to the discovery of a new series of 2,4-disubstituted quinolines as allosteric enhancers of the adenosine  $A_3$  receptor. In particular compound **27** (LUF6096) was able to decrease the dissociation rate of  $^{125}\text{I}$ -AB-MECA to a similar extent as **34**. In addition, compound **27** had a decreased orthosteric effect at both the adenosine  $A_3$  and  $A_1$  receptors. In a functional assay, **27** and **34** increased the intrinsic efficacy of the agonist CI-IB-MECA. Moreover, the presence of **27** significantly increased the potency of CI-IB-MECA, unlike **34**.

## Experimental Section

**Chemistry: Material and Methods.** Microwave-assisted chemistry was performed on an Emrys Optimizer with Emrys Optimizer software. For the reactions, vials with a volume of 2–5 mL were used.  $^1\text{H}$  NMR spectra were measured at 200 MHz with a Bruker AC 200 or Bruker DMX 600 spectrometer.  $^{13}\text{C}$  NMR spectra were measured at 50 or 150 MHz. Chemical shifts for  $^1\text{H}$  and  $^{13}\text{C}$  are given in ppm ( $\delta$ ) relative to tetramethylsilane (TMS) as internal standard, and 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.

**General Procedure for the Preparation of 2,4-Substituted Quinolines (16–33).** **Method A.** Compounds **10–15** were dissolved/suspended in absolute ethanol (1.5 mmol/2.5 mL), and the appropriate amines (3 equiv) were added. The mixture was heated

in the microwave at 140 °C for 80 min. After the reaction was completed, ethanol was evaporated and the residue was dissolved in DCM (100 mL) and washed with 1 M NaOH (3 × 100 mL). The organic layer was dried on MgSO<sub>4</sub>. The products were purified by column chromatography and recrystallized.

**Method B.** Compounds **10–15** and the appropriate amines (10 equiv) were heated in the microwave without any solvent at 180 °C for 90 min. After the reaction was completed, the reaction mixture was dissolved in DCM (100 mL) and washed with water (2 × 50 mL) and brine (1 × 50 mL). The organic layer was dried on MgSO<sub>4</sub>. The products were purified by column chromatography and recrystallized.

***N*-(2-Anilinoquinolin-4-yl)cyclopentanecarboxamide (16).** **Method A.** An amount of 0.36 mmol of *N*-(2-bromoquinolin-4-yl)cyclopentanecarboxamide (**10**) was used, and the eluent was 3–10% MeOH in DCM. The product was recrystallized from methanol to give yellow crystals. Yield: 0.039 g, 33%. MS (ESI) *m/z*: 331.2 [M + H]<sup>+</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.72–2.04 (m, 8H, 4CH<sub>2</sub>), 2.80–2.98 (m, 1H, CH), 6.93 (br s, 1H, NH), 7.07 (t, 1H, *J* = 5.74, 8.06 Hz, Ar), 7.25–7.41 (m, 3H, Ar), 7.57–7.67 (m, 5H, m, Ar), 7.78–7.83 (m, 1H, Ar), 8.01 (s, 1H, NH). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 25.63, 30.18, 46.89, 100.90, 116.18, 118.36, 119.73, 122.50, 127.25, 129.46, 139.77, 140.96, 147.54, 154.51, 174.87. Anal. (C<sub>21</sub>H<sub>21</sub>N<sub>3</sub>O·0.3H<sub>2</sub>O) C, H, N.

***N*-(2-[(3,4-Dichlorophenyl)amino]quinolin-4-yl)cyclohexanecarboxamide (27).** **Method B.** An amount of 0.17 mmol of *N*-(2-chloroquinolin-4-yl)cyclohexanecarboxamide (**12**) was used, and the eluent was 1% MeOH in DCM. The product was recrystallized from ethyl acetate to give white crystals. Yield: 0.16 g, 43%. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.23–2.17 (m, 10H, 5CH<sub>2</sub>), 2.35–2.50 (m, 1H, CH), 6.77 (bs, 1H, NH), 7.32–7.41 (m, 2H, Ar), 7.49 (dd, 1H, *J* = 6.22, 2.56 Hz, Ar), 7.58–7.67 (m, 2H, Ar), 7.84–7.88 (m, 2H, Ar, NH), 7.95 (s, 1H, Ar), 8.09 (d, 1H, *J* = 2.56 Hz, Ar). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) δ 25.23, 25.48, 29.27, 38.30, 44.46, 103.69, 117.58, 118.24, 119.03, 121.55, 122.00, 122.55, 127.07, 129.67, 130.28, 130.80, 141.89, 141.98, 147.50, 154.26, 175.87. Anal. (C<sub>22</sub>H<sub>21</sub>Cl<sub>2</sub>N<sub>3</sub>O·0.5H<sub>2</sub>O) C, H, N.

**Biology: Material and Methods.** NECA, Cl-IB-MECA, R-PIA, and DPCPX were purchased from Sigma Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Compound **34** was synthesized in our laboratory as described previously.<sup>15</sup> <sup>125</sup>I-AB-MECA (specific activity 2200 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Groningen, The Netherlands). [<sup>3</sup>H]ZM241385 (specific activity 17 Ci/mmol) and [<sup>3</sup>H]MRS1754 (specific activity 37 Ci/mmol) were obtained from Tocris Cookson, Ltd. (U.K.), and CGS 21680 was a gift from Dr. R. A. Lovell (Novartis, Summit, NJ). CHO (Chinese hamster ovary) cells stably expressing the human adenosine A<sub>1</sub> (CHOA<sub>1</sub>) or A<sub>3</sub> receptor (CHOA<sub>3</sub>) were obtained from Dr. A. Townsend-Nicholson (University College London, U.K.) and Dr. K.-N. Klotz (University of Würzburg, Germany), respectively. HEK293 (human embryonic kidney) cells stably expressing the human adenosine A<sub>2A</sub> receptor (HEK293hA<sub>2A</sub>) were obtained from Dr. J. Wang (Biogen Idec, Cambridge, MA). CHO cells stably expressing the human adenosine A<sub>2B</sub> receptor and a secreted placental alkaline phosphatase (SPAP) reporter gene (CHOA<sub>2B</sub>\_SPAP) were obtained from Dr. S. J. Dowell (GSK, Stevenage, U.K.). All other chemicals and cell culture materials were obtained from standard commercial sources.

**Cell Culture and Membrane Preparation.** CHOA<sub>1</sub> cells were cultured as described previously.<sup>29</sup> HEK293hA<sub>2A</sub> cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) normal adult bovine serum, streptomycin (50 μg/mL), penicillin (50 IU/mL), and G418 (0.5 mg/mL) at 37 °C in 7% CO<sub>2</sub>. The cells were subcultured twice weekly at a ratio of 1:8. CHOA<sub>2B</sub>\_SPAP cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1) supplemented with 10% (v/v) normal adult bovine serum, streptomycin (50 μg/mL), penicillin (50 IU/mL), G418 (1.0 mg/mL), and hygromycin B (0.4 mg/mL) at 37 °C in 5% CO<sub>2</sub>. The cells were subcultured twice weekly at a ratio of 1:10. CHOA<sub>3</sub> cells were grown in culture

medium consisting of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1) supplemented with 10% (v/v) normal adult bovine serum, streptomycin (50 μg/mL), penicillin (50 IU/mL), and G418 (0.4 mg/mL) at 37 °C in 5% CO<sub>2</sub>. The cells were subcultured twice weekly at a ratio of 1:20. All cell membranes were prepared as described previously.<sup>29</sup>

**Radioligand Displacement Assays. Adenosine A<sub>1</sub> and A<sub>3</sub> Receptor.** Membrane aliquots containing 20 μg (A<sub>1</sub>) or 10 μg of protein (A<sub>3</sub>) were incubated in a total volume of 100 μL assay buffer (50 mM Tris HCl, pH 8.0, supplemented with 10 mM MgCl<sub>2</sub>, 1 mM EDTA, and 0.01% (w/v) CHAPS) at 25 °C for 60 min (A<sub>1</sub>) or 120 min (A<sub>3</sub>). Displacement experiments were performed using 10 μM competing ligand in the presence of 45 000 cpm (~0.1 nM) <sup>125</sup>I-AB-MECA. Nonspecific binding was determined in the presence of 10 μM DPCPX (A<sub>1</sub>) or 100 μM R-PIA (A<sub>3</sub>). Incubations were terminated by dilution with ice-cold assay buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed three times with 2 mL of ice-cold buffer. Filter-bound radioactivity was determined in a γ-counter (Wizard 1470, PerkinElmer Life and Analytical Sciences).

**Adenosine A<sub>2A</sub> Receptor.** Membrane aliquots containing 30 μg of protein were incubated in a total volume of 200 μL of assay buffer (50 mM Tris-HCl, pH 7.4) at 25 °C for 120 min. Displacement experiments were performed using 10 μM competing ligand in the presence of ~1.7 nM [<sup>3</sup>H]ZM241385. Nonspecific binding was determined in the presence of 100 μM CGS 21680. Incubations were terminated, and samples were obtained as described under Adenosine A<sub>1</sub> and A<sub>3</sub> Receptor. Filter-bound radioactivity was determined in a β-counter (Tri-Carb 2900 TR, PerkinElmer Life and Analytical Sciences) after addition of 3.5 mL of PerkinElmer Emulsifier Safe.

**Adenosine A<sub>2B</sub> Receptor.** Membrane aliquots containing 20 μg of protein were incubated in a total volume of 100 μL of assay buffer (50 mM Tris-HCl, pH 7.4, supplemented 0.1% (w/v) CHAPS) at 25 °C for 60 min. Displacement experiments were performed using 10 μM competing ligand in the presence of ~1.2 nM [<sup>3</sup>H]MRS1754. Nonspecific binding was determined in the presence of 1 mM NECA. Incubations were terminated, and samples were obtained and analyzed as described under Adenosine A<sub>2A</sub> Receptor.

**Radioligand Kinetic Dissociation Assays.** Dissociation experiments were performed by preincubating membrane aliquots containing 20 μg (A<sub>1</sub>) or 10 μg of protein (A<sub>3</sub>) in a total volume of 100 μL of assay buffer at 25 °C for 60 min (A<sub>1</sub>) or 120 min (A<sub>3</sub>) with 45 000 cpm (~0.1 nM) <sup>125</sup>I-AB-MECA. After preincubation, dissociation was initiated by addition of 10 μM DPCPX (A<sub>1</sub>) or 10 μM Cl-IB-MECA (A<sub>3</sub>) in the absence (control) or presence of allosteric modulators in a volume of 5 μL, of which 25% (v/v) is DMSO. The amount of radioligand still bound to the receptor was measured after 90 (A<sub>1</sub>) and 120 min (A<sub>3</sub>) of dissociation. The obtained amount of radioligand binding determined at control conditions was set at 100%. Incubations were terminated and samples were obtained and analyzed as described under Radioligand Displacement Assays.

**cAMP Determination.** Intracellular cAMP levels were measured using a LANCE cAMP 384 kit (PerkinElmer, The Netherlands) as described previously.<sup>30</sup> In short, to each well different concentrations of Cl-IB-MECA in stimulation buffer (PBS with 5 mM HEPES, pH 7.4, supplemented with 0.1% BSA, rolipram (50 μM), and cilostamide (50 μM)) were added in a volume of 5 μL in the absence (control) or presence of allosteric modulators. Then 4.5 μL of CHOA<sub>3</sub> cell suspension in stimulation buffer was seeded into a 384-well plate (approximately 5000 cells/well), which was followed by incubation for 15 min at room temperature. Subsequently, 2.5 μL of forskolin (1 μM) was added and the mixture was incubated for 30 min at room temperature. Finally, 3 h after addition of detection mix (6 μL) and cAMP antibody solution (6 μL), intracellular cAMP levels were measured using a TR-FRET assay on a Victor spectrometer (PerkinElmer, The Netherlands) according to instructions of the supplier.

**Data Analysis.** All binding data was analyzed using the nonlinear regression curve-fitting program GraphPad Prism, version 5.00 (GraphPad Software Inc., San Diego, CA). Dissociation rate constants,  $k_{\text{off}}$ , were obtained by computer analysis of the exponential decay of the percentage of  $^{125}\text{I}$ -AB-MECA bound to the receptor.  $\text{EC}_{50}$  values were directly obtained from the dose–response curves. All values obtained are mean values of three independent experiments performed in duplicate.

**Supporting Information Available:** Experimental details of the synthesis of the compounds described in this paper, their  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectroscopic data, and their elemental analysis results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (1) Fredholm, B. B.; IJzerman, A. P.; Jacobson, K. A.; Klotz, K. N.; Linden, J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **2001**, *53*, 527–552.
- (2) Ralevic, V.; Burnstock, G. Receptors for purines and pyrimidines. *Pharmacol. Rev.* **1998**, *50*, 413–492.
- (3) Soudijn, W.; van Wijngaarden, I.; IJzerman, A. P. Medicinal chemistry of adenosine A<sub>1</sub> receptor ligands. *Curr. Top. Med. Chem.* **2003**, *3*, 355–367.
- (4) Cristalli, G.; Cacciari, B.; Dal Ben, D.; Lambertucci, C.; Moro, S.; Spalluto, G.; Volpini, R. Highlights on the development of A<sub>2A</sub> adenosine receptor agonists and antagonists. *ChemMedChem* **2007**, *2*, 260–281.
- (5) Beukers, M. W.; Meurs, I.; IJzerman, A. P. Structure–affinity relationships of adenosine A<sub>2B</sub> receptor ligands. *Med. Res. Rev.* **2006**, *26*, 667–698.
- (6) Müller, C. E. Medicinal chemistry of adenosine A<sub>3</sub> receptor ligands. *Curr. Top. Med. Chem.* **2003**, *3*, 445–462.
- (7) May, L. T.; Leach, K.; Sexton, P. M.; Christopoulos, A. Allosteric modulation of G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 1–51.
- (8) Christopoulos, A.; Kenakin, T. G protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.* **2002**, *54*, 323–374.
- (9) Soudijn, W.; Van Wijngaarden, I.; IJzerman, A. P. Allosteric modulation of G protein-coupled receptors: perspectives and recent developments. *Drug Discovery Today* **2004**, *9*, 752–758.
- (10) Gao, Z. G.; Melman, N.; Erdmann, A.; Kim, S. G.; Müller, C. E.; IJzerman, A. P.; Jacobson, K. A. Differential allosteric modulation by amiloride analogues of agonist and antagonist binding at A<sub>1</sub> and A<sub>3</sub> adenosine receptors. *Biochem. Pharmacol.* **2003**, *65*, 525–534.
- (11) Gao, Z. G.; IJzerman, A. P. Allosteric modulation of A<sub>2A</sub> adenosine receptors by amiloride analogues and sodium ions. *Biochem. Pharmacol.* **2000**, *60*, 669–676.
- (12) Bruns, R. F.; Fergus, J. H. Allosteric enhancement of adenosine A<sub>1</sub> receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol. Pharmacol.* **1990**, *38*, 939–949.
- (13) Gao, Z. G.; Van Muijlwijk-Koezen, J. E.; Chen, A.; Müller, C. E.; IJzerman, A. P.; Jacobson, K. A. Allosteric modulation of A<sub>3</sub> adenosine receptors by a series of 3-(2-pyridinyl)isoquinoline derivatives. *Mol. Pharmacol.* **2001**, *60*, 1057–1063.
- (14) Gao, Z. G.; Kim, S. G.; Soltysiak, K. A.; Melman, N.; IJzerman, A. P.; Jacobson, K. A. Selective allosteric enhancement of agonist binding and function at human A<sub>3</sub> adenosine receptors by a series of imidazoquinoline derivatives. *Mol. Pharmacol.* **2002**, *62*, 81–89.
- (15) Göblyös, A.; Gao, Z. G.; Brussee, J.; Connestari, R.; Santiago, S. N.; Ye, K.; IJzerman, A. P.; Jacobson, K. A. Structure–activity relationships of new 1*H*-imidazo[4,5-*c*]quinolin-4-amine derivatives as allosteric enhancers of the A<sub>3</sub> adenosine receptor. *J. Med. Chem.* **2006**, *49*, 3354–3361.
- (16) Ochiai, E. Recent Japanese work on the chemistry of pyridine 1-oxide and related compounds. *J. Org. Chem.* **1953**, *18*, 534–551.
- (17) Zhong, P.; Guo, S. R.; Song, C. S. A simple and efficient method for the preparation of heterocyclic *N*-oxide. *Synth. Commun.* **2004**, *34*, 247–253.
- (18) Taylor, E. C.; Crovetti, A. J. 3-Methyl-4-nitropyridine-1-oxide. *Org. Synth.* **1956**, *36*, 53–55.
- (19) Hamana, M.; Hoshida, Y.; Kaneda, K. A new deoxidation of aromatic tertiary amine oxides. Reaction of 4-nitroquinoline 1-oxide with phosphorus bromide. *Chem. Abstr.* **1956**, *51*, 6639.
- (20) Wozniak, M.; Grzegozek, M. Amination of 4-nitroquinoline with liquid methylamine potassium permanganate. *Chem. Heterocycl. Compd.* **1998**, *34*, 837–840.
- (21) Kornblum, N.; Fishbein, L. The chemistry of aliphatic and alicyclic nitro compounds. 8. The Reduction of Optically Active 2-Nitrooctane and Alpha-Phenylnitroethane. *J. Am. Chem. Soc.* **1955**, *77*, 6266–6269.
- (22) Chang, L. C. W.; Spanjersberg, R. F.; Von Frijtag Drabbe Künzel, J. K.; Mulder-Krieger, T.; Van den Hout, G.; Beukers, M. W.; Brussee, J.; IJzerman, A. P. 2,4,6-Trisubstituted pyrimidines as a new class of selective adenosine A<sub>1</sub> receptor antagonists. *J. Med. Chem.* **2004**, *47*, 6529–6540.
- (23) Osborne, A. G.; Buley, J. M.; Clarke, H.; Dakin, R. C. H.; Price, P. I. 2,4-Dihalogenoquinolines. Synthesis, orientation effects and H-1 and C-13 NMR spectral studies. *J. Chem. Soc., Perkin Trans 1* **1993**, *1*, 2747–2755.
- (24) Von Büchi, J.; Hurni, H.; Lieberherr, R. Die Tuberkulostatische Wirkung von 2-oxy-4-amino-chinolin-derivaten. *Helv. Chim. Acta* **1949**, *32*, 1806–1816.
- (25) Wojahn, H. Untersuchungen über den Zusammenhang von chemischer constitution und anästhesierender Wirkung bei 2-alkoxy-chinolin-derivaten. *Arch. Pharm.* **1936**, *274*, 83–106.
- (26) Neubig, R. R.; Spedding, M.; Kenakin, T.; Christopoulos, A. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **2003**, *55*, 597–606.
- (27) Urwyler, S.; Mosbacher, J.; Lingenhöhl, K.; Heid, J.; Hofstetter, K.; Froestl, W.; Bettler, B.; Kaupmann, K. Positive allosteric modulation of native and recombinant gamma-aminobutyric acid(B) receptors by 2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930) and its aldehyde analog CGP13501. *Mol. Pharmacol.* **2001**, *60*, 963–71.
- (28) Urwyler, S.; Pozza, M. F.; Lingenhöhl, K.; Mosbacher, J.; Lampert, C.; Froestl, W.; Koller, M.; Kaupmann, K. *N,N'*-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) and structurally related compounds: novel allosteric enhancers of gamma-aminobutyric acid B receptor function. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 322–330.
- (29) Heitman, L. H.; Mulder-Krieger, T.; Spanjersberg, R. F.; von Frijtag Drabbe Künzel, J. K.; Dalpiaz, A.; IJzerman, A. P. Allosteric modulation, thermodynamics and binding to wild-type and mutant (T277A) adenosine A<sub>1</sub> receptors of LUF5831, a novel nonadenosine-like agonist. *Br. J. Pharmacol.* **2006**, *147*, 533–541.
- (30) Van Veldhoven, J. P.; Chang, L. C.; Von Frijtag Drabbe Künzel, J. K.; Mulder-Krieger, T.; Struensee-Link, R.; Beukers, M. W.; Brussee, J.; IJzerman, A. P. A new generation of adenosine receptor antagonists: from di- to trisubstituted aminopyrimidines. *Bioorg. Med. Chem.* **2008**, *16*, 2741–2752.

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