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Selective Allosteric Enhancement of Agonist Binding and Function at Human A_3 Adenosine Receptors by a Series of Imidazoquinoline Derivatives

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ABSTRACT

We have identified a series of 1H-imidazo-[4,5-*c*]quinolines as selective allosteric enhancers of human A_3 adenosine receptors. Several of these compounds potentiated both the potency and maximal efficacy of agonist-induced responses and selectively decreased the dissociation of the agonist N^6-(4-amino-3-[^{125}I]iodobenzyl)-5′-*N*-methylcarboxamidoadenosine from human A_3 adenosine receptors. There was no effect on the dissociation of the antagonist [^{3}H]8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1H-imidazo[2.1-*i*]purin-5-one (PSB-11) from the A_3 receptors, as well as [^{3}H]N^6-[(*R*)-phenylisopropyl]adenosine from rat brain A_1 receptors and [^{3}H]2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine from rat striatal A_{2A} receptors, suggesting the selective enhancement of agonist binding at A_3 receptors. The analogs were tested as antagonists of competitive binding at human A_3 receptors, and K_i values ranging from 120 nM to 101 μM were observed; as for many allosteric modulators of G protein-coupled receptors, an orthosteric effect was also present. The most promising leads from the present set of analogs seem to be the 2-cyclopentyl-1H-imidazo[4,5-*c*]quinoline derivatives, of which the 4-phenylamino analog DU124183 had the most favorable degree of allosteric modulation versus receptor antagonism. The inhibition of forskolin-stimulated cyclic AMP accumulation in intact cells that express human A_3 receptors was employed as a functional index of A_3 receptor activation. The enhancer DU124183 caused a marked leftward shift of the concentration-response curve of the A_3 receptor agonists in the presence of antagonist and, surprisingly, a potentiation of the maximum agonist efficacy by approximately 30%. Thus, we have identified a novel structural lead for developing allosteric enhancers of A_3 adenosine receptors; such enhancers may be useful for treating brain ischemia and other hypoxic conditions.

In addition to the orthosteric ligand binding site for competitive antagonists and agonists, the A_3 adenosine receptor contains an allosteric binding site (Gao et al., 2001). An allosteric ligand and a competitive ligand can bind to a given receptor simultaneously and modulate the affinity of the other. In the case of the ion channel-coupled $GABA_A$ receptor, diazepam enhances the endogenous neurotransmitter GABA via an allosteric site (Macdonald and Olsen, 1994), thus providing therapeutic efficacy, whereas the direct-acting agonists are not used clinically because of the side effects. In the ion channel-coupled nicotinic receptors, galanthamine, which is an acetylcholinesterase inhibitor as well as an allosteric modulator at nicotinic receptor sites potentiating nicotinic cholinergic neurotransmission, has recently been ex-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DU124183, 2-cyclopentyl-4-phenylamino-1H-imidazo[4,5-*c*]quinoline; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; CGS15943, 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-*c*]quinazoline; NECA, 5′-*N*-ethylcarboxamidoadenosine; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine; MRS1220, *N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl]benzeneacetamide; I-AB-MECA, N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; PSB-11, 8-ethyl-4-methyl2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1H-imidazo[2.1-*i*]purin-5-one; (*R*)-PIA, N^6-[(*R*)-phenylisopropyl]adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CGS21680, 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine; ZM241385, (4-(2-[7-amino-2-(2-furyl)(triazolo{2,3-*a*}-[1,3,5]triazin-5-ylamino]ethyl)phenol); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DU124482, 4-cyclopentylamino-2-phenyl-1*H*-imidazo[4,5-*c*]quinoline; DU124480, 2-phenyl-4-phenyloxy-1*H*-imidazol[4,5-*c*]quinoline; DU124276, 4-chloro-1*H*-imidazo[4,5-*c*]quinoline; DU124182, 2-cyclopentyl-4-phenyloxy-1H-imidazo [4,5-*c*]quinoline; DU124184, 2-cyclopentyl-4-cyclopentylamino-1H-imidazo[4,5-*c*]quinoline; DU124483, 4-amino-2-phenyl-1*H*-imidazo[4,5-*c*]quinoline; DU124185, 4-amino-2-cyclopentyl-1*H*-imidazo[4,5-*c*]quinoline; MRS1898, (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol; VUF5455, 4-methoxy-*N*-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide; CADO, 2-chloroadenosine; PD81723, 2-amino-4,5-dimethyl-3-thienyl-[3-(trifluoromethyl)phenyl]methanone.

tensively and successfully used in clinical trials and also showed satisfactory therapeutic effects in Alzheimer's disease (Olin and Schneider, 2001). Thus, the presence of the allosteric site provided a new target for drug discovery.

Although no allosteric modulator for a G protein-coupled receptor has been used clinically so far, several lines of evidence suggested that allosteric modulators might have advantages over classical ligands from the therapeutic point of view (Birdsall et al., 1995; Linden, 1997; Kobilka, 2000; Pin et al., 2001). The therapeutic effects of agonists may be limited by receptor desensitization. Allosteric modulators, by potentiating the effects of the endogenous agonists, may be more selective compared with the classical agonists (Birdsall et al., 1995; Bhattacharya and Linden, 1996; Linden, 1997). The allosteric site may not have been conserved during evolution as strictly as the orthosteric ligand binding site. Currently available muscarinic drugs show only modest subtype selectivity, probably because of the strict conservation of sequence in regions considered to bind agonists (Hulme et al., 1990). Even if the allosteric sites of different receptor subtypes are very similar, only a small intersubtype difference in protein sequence might alter the characteristic cooperativity at one subtype over another subtype. For example, the design of subtype-selective agonists or antagonists for any single subtype of G protein-coupled muscarinic receptors has proven elusive. However, by targeting the allosteric site on muscarinic receptors, compounds with much greater subtype selectivity have been identified (Birdsall et al., 1999). One such allosteric modulator is the alkaloid brucine, which is capable of selectively enhancing the effects of acetylcholine at only m1 receptors, whereas *N*-chloromethylbrucine enhances acetylcholine actions at only m3 receptors. Brucine *N*-oxide enhances acetylcholine binding at both m3 and m4 receptors. By targeting the allosteric site, the first subtype-selective non–amino acid-like antagonists for $mGlu_5$ receptors have also been recently identified (Spooren et al., 2001).

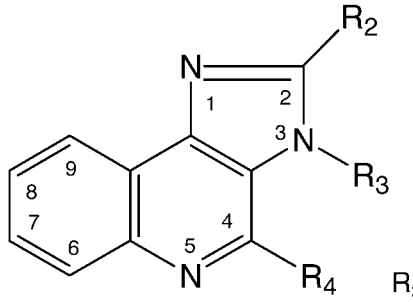
We have recently reported that a series of 3-(2-pyridinyl)isoquinoline derivatives allosterically enhanced agonist binding at A_3 adenosine receptors (Gao et al., 2001). In the course of screening potential allosteric modulators for A_3 receptors, we found that a series of 1H-imidazo-[4,5-*c*]quinolines (Fig. 1), including DU124183, enhanced both agonist binding and function at A_3 receptors. Herein we describe the characteristics of allosteric modulation of A_3 adenosine receptors by these compounds employing both dissociation kinetics and a cyclic AMP functional assay in intact Chinese hamster ovary (CHO) cells expressing human A_3 adenosine receptors.

Experimental Procedures

Materials. CPA, CGS15943, NECA, Cl-IB-MECA, and MRS1220 were purchased from Sigma (St. Louis, MO). [^{125}I]I-AB-MECA; 2000 Ci/mmol), [^{3}H]PSB-11 (53 Ci/mmol), [^{3}H](*R*)-PIA (34 Ci/mmol), [^{3}H]DPCPX (120 Ci/mmol), and [^{3}H]CGS21680 (47 Ci/mmol) were from Amersham Bioscience (Buckinghamshire, UK). [^{3}H]ZM241385 was from Tocris (Ballwin, MO). 1H-Imidazo-[4,5-*c*]quinolines were from the Leiden/Amsterdam Center for Drug Research (Leiden, The Netherlands).

Cell Culture and Membrane Preparation. CHO cells expressing recombinant human A_3 adenosine receptors were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 800 μg/ml geneticin. 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 8.0, containing 10 mM $MgCl_2$, 1 mM EDTA, and 0.1 mg/ml CHAPS. The suspension was homogenized with an electric homogenizer for 10 s, and was then recentrifuged at 16,000*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. Striatal and forebrain tissues from Wistar rats were homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.4, using an electric homogenizer. The homogenate was centrifuged at 20,000*g* for 10 min at 4°C, and the pellet was washed in fresh buffer. The final pellet was stored at −80°C until the binding experiments. The protein concentration was measured using the assay of Bradford (1976).

Dissociation Kinetics of [^{125}I]I-AB-MECA and [^{3}H]PSB-11 from A_3 Adenosine Receptors. The dissociation of [^{125}I]I-AB-MECA was measured as follows. Membranes (20 μg) were preincubated at 25°C with 1.0 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 by filtration through Whatman GF/B glass-fiber filters under reduced pressure using a MT-24 cell harvester (Brandel, Gaithersburg, MD), and radioactivity was determined using a gamma-counter (5500B; Beckman Coulter, Fullerton, CA).



R_2 =	R_4 =	Compound
Cp	O-Ph	DU124182
Cp	NH-Ph	DU124183
Cp	NH-Cp	DU124184
Cp	NH_2	DU124185
H	Cl	DU124276
H	NH-Ph	DU124278
H	O-Ph	DU124277
Ph	O-Ph	DU124280
Ph	NH-Ph	DU124481
Ph	NH-Cp	DU124482
Ph	NH_2	DU124483
Cp	O-Ph(R_3 = CH_3)	DU124545

Fig. 1. Chemical structures of the imidazo-[4,5-*c*]quinoline derivatives used in the present study. Cp = cyclopentyl; Ph = phenyl.

For competitive binding experiments, each tube contained 50 μl of membrane suspension, 25 μl of [^{125}I]I-AB-MECA (final concentration 1.0 nM), and 25 μl of increasing concentrations of test compounds in Tris-HCl buffer (50 mM, pH 8.0). For the dissociation of antagonist [i.e., [^{3}H]PSB-11 (Müller et al., 2002)] from A_3 adenosine receptors, membranes (80 μg) were preincubated with 8 nM [^{3}H]PSB-11 at 25°C in a total assay volume of 200 μl for 60 min. The dissociation was initiated by addition of 3 μM Cl-IB-MECA with or without tested compounds. The time course of dissociation was measured by rapid filtration at appropriate time intervals. Nonspecific binding was measured after an incubation of 60 min in the presence of 3 μM Cl-IB-MECA.

Dissociation of [^{3}H](*R*)-PIA and [^{3}H]DPCPX from A_1 Adenosine Receptors. Binding of 1 nM [^{3}H](*R*)-PIA to A_1 adenosine receptors in rat forebrain membranes (80 μg/tube) was carried out at 37°C for 90 min in 50 mM Tris-HCl buffer, pH 7.7, containing 10 mM $MgCl_2$ in a total assay volume of 400 μl. Binding of [^{3}H]DPCPX to A_1 adenosine receptors in rat forebrain membranes (60 μg/tube) was carried out at 25°C for 60 min in 50 mM Tris-HCl buffer, pH 7.4, in a total assay volume of 400 μl. The dissociation was begun by addition of 10 μM CPA with or without tested compounds. Nonspecific binding was determined using 10 μM CPA. Samples were filtered after incubation at the time points indicated.

Dissociation of [^{3}H]CGS21680 and [^{3}H]ZM241385 from A_{2A} Adenosine Receptors. Rat striatal membranes (80 μg/tube) were incubated with 15 nM [^{3}H]CGS21680 at 25°C for 90 min in 400 μl of 50 mM Tris-HCl, pH 7.7, containing 10 mM $MgCl_2$. Dissociation was started by the addition of 10 μM NECA in the presence and absence of tested compounds. For the dissociation of [^{3}H]ZM241385, the procedures were similar to that of [^{3}H]CGS21680.

Cyclic AMP Accumulation Assay. Cyclic AMP levels were measured with a competitive protein binding method (Nordstedt and Fredholm, 1990; Post et al., 2000). CHO cells that expressed recombinant human A_3 adenosine receptors were harvested by trypsinization. After centrifugation and resuspension in medium, cells were deposited in 24-well plates in volumes of 1 ml. After 24 h, the medium was removed and cells were washed three times with 1 ml of Dulbecco's modified Eagle's medium 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 the incubation was continued for an additional 15 min. The reaction was terminated by removal of the supernatant, and cells were lysed upon the addition of 200 μl of ice-cold 0.1 M HCl. The cell lysate was resuspended and stored at −20°C. For determination of cyclic AMP production, protein kinase A 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 (0–80 nM 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 counter.

Statistical Analysis. Results from cyclic AMP assay and binding parameters were analyzed by Prism software (GraphPad, San Diego, CA). IC_{50} values obtained from competition curves were converted to K_i values by using the Cheng and Prusoff equation (1973). Data were expressed as mean ± S.E.

Results

Time Course of Agonist [^{125}I]I-AB-MECA and Antagonist [^{3}H]PSB-11 Dissociation from A_3 Receptors in the Absence and Presence of Potential Allosteric Modulators. The dissociation of [^{125}I]I-AB-MECA from human A_3 receptors was measured in the absence and presence of test compounds (Fig. 1, Table 1). DU124183 significantly decreased the dissociation rate, whereas the isomeric compound DU124482 influenced the rate of dissociation only slightly (Fig. 2). The dissociation rates in the absence and presence of 10 μM DU124183 were 0.056 ± 0.008 and 0.030 ± 0.006 min^{-1}, respectively, which were significantly different ($p < 0.05$). By comparison, the nonselective adenosine receptor antagonist CGS15943 (30 μM) and the A_1 receptor enhancer of agonist action, PD81723 (30 μM) did not affect the dissociation rate at A_3 receptors (Table 1). The dissociation rates in the absence and presence of the imidazoquinoline derivatives and the residual radioligand binding remaining after 45 min of dissociation are summarized in Table 1. For the most potent allosteric modulators, the percentage of residual [^{125}I]I-AB-MECA binding after dissociation of 45 min was roughly twice the percentage in the absence of test compounds.

In contrast to its effect on agonist dissociation from the A_3 receptor, DU124183 and DU124482 did not influence the dissociation rate of the A_3 antagonist [^{3}H]PSB-11 (Fig. 3). The dissociation rates (k_{-1}) in the presence of DU124183 and DU124482 were 0.33 ± 0.05 and 0.33 ± 0.07 min^{-1}, respectively, which were not significantly different from the k_{-1} value (k_{-1} = 0.31 ± 0.04 min^{-1}) in the absence of allosteric modulators ($P > 0.05$).

Concentration-Dependent Effect of DU124183 on Agonist Radioligand [^{125}I]I-AB-MECA from the A_3 Receptor. To further demonstrate the allosteric effects of DU124183, we observed the concentration-dependent effects of these compounds on the dissociation of [^{125}I]I-AB-MECA from the human A_3 receptor. Figure 4 shows the influence of

TABLE 1

Allosteric enhancing effects (at 10 μM, indicated by effects on dissociation) and orthosteric binding inhibition constants (K_i) of imidazoquinolinamine derivatives at human A_3 receptors expressed in CHO cells. For the dissociation experiment, [^{125}I]I-AB-MECA (1 nM) was preincubated with membranes (20 μg of protein) expressing human A_3 receptors at 25°C for 60 min. Dissociation was initiated by adding 3 μM Cl-IB-MECA, in either the absence or presence of test compounds. The concentration of the imidazoquinolinamine derivatives used in the dissociation experiment was 10 μM, and other compounds were used at 30 μM, as indicated. Binding is the percentage of [^{125}I]I-AB-MECA binding remaining after dissociation for 45 min in control membranes and in the presence of test compounds. The initial occupancy (B_0) of the receptor corresponded to approximately 50% of the receptors present. Under these conditions, less than 5% of total [^{125}I]I-AB-MECA was receptor-bound. K_i, the inhibition constant, was calculated from the IC_{50} value using the Cheng-Prusoff equation (1973), assuming a K_D value of 0.99 nM. Results are expressed as mean ± S.E.M. from three independent experiments.

Compound	k_{-1}	Binding	K_i
	min^{-1}	*%*	*μM*
Control	0.056 ± 0.008	26 ± 4	
DU124182	0.031 ± 0.007*	42 ± 3*	0.31 ± 0.07
DU124183	0.030 ± 0.006*	48 ± 5*	0.82 ± 0.13
DU124184	0.042 ± 0.004*	45 ± 2*	0.32 ± 0.01
DU124185	0.061 ± 0.006	27 ± 4	5.6 ± 2.1
DU124276	0.050 ± 0.006	38 ± 5*	0.16 ± 0.02
DU124277	0.053 ± 0.012	28 ± 4	2.2 ± 0.6
DU124278	0.091 ± 0.007*	21 ± 2*	1.3 ± 0.4
DU124480	0.052 ± 0.008	27 ± 3	0.12 ± 0.01
DU124481	0.074 ± 0.006*	22 ± 2*	0.81 ± 0.08
DU124482	0.045 ± 0.007*	37 ± 3*	0.30 ± 0.01
DU124483	0.057 ± 0.007	26 ± 2	0.39 ± 0.13
DU124545	0.054 ± 0.013	28 ± 3	101 ± 29
PD81723 (30 μM)	0.055 ± 0.008	26 ± 3	N.D.
CGS15943 (30 μM)	0.053 ± 0.008	27 ± 4	0.10 ± 0.03
Caffeine (30 μM)	0.057 ± 0.012	27 ± 3	N.D.

N.D., not determined.
* $P < 0.05$ compared with control.

increasing concentrations of DU124183 on the dissociation of [^{125}I]I-AB-MECA in the presence of 3 μM Cl-IB-MECA. Dissociation was allowed to proceed for 45 min before the reaction was terminated by filtration. DU124183 decreased the dissociation rate in a concentration-dependent manner.

Dissociation of [^{3}H]R.-PIA and [^{3}H]DPCPX from A_1 Adenosine Receptors. In contrast to the enhancement of A_3 receptor agonist binding, DU124183 and DU124482 did not enhance the binding of A_1 receptor agonist (Fig. 5a). The dissociation rates in the absence (0.14 ± 0.02 min^{-1}) and in the presence of 10 μM DU124183 and DU124482 (0.14 ± 0.03 and 0.15 ± 0.02 min^{-1}, respectively) were not significantly different ($p > 0.05$). Similar to its effects on agonist dissociation, DU124183 did not influence the dissociation of [^{3}H]D-PCPX from A_1 adenosine receptors (Fig. 5b). This contrasts with the effect of amiloride, which increased the dissociation rate of [^{3}H]DPCPX from A_1 adenosine receptors (Fig. 5b).

Dissociation of [^{3}H]CGS21680 and [^{3}H]ZM241385 from Rat A_{2A} Adenosine Receptors. Similar to their effects on A_1 receptors, DU124183 and DU124482 did not enhance the dissociation rate of [^{3}H]CGS21680 from A_{2A} receptors (Fig. 6a). The dissociation rates in the absence and

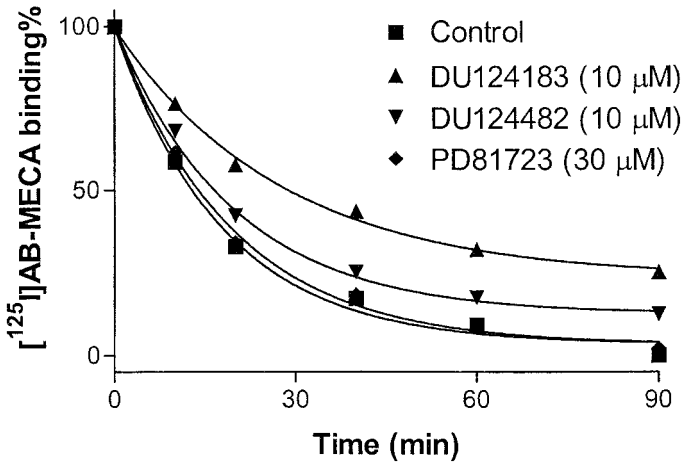


Fig. 2. Effects of DU124183 and DU124482 on the dissociation of [^{125}I]I-AB-MECA from human A_3 adenosine receptors expressed in CHO cells. [^{125}I]I-AB-MECA (1.0 nM) was preassociated with the cell membranes during a 1-h incubation at 25°C in a total assay volume of 100 μl. Nonspecific binding was determined in parallel by addition of 3 μM Cl-IB-MECA before the preassociation. After the 1-h preassociation, dissociation was initiated by addition of Cl-IB-MECA (final concentration, 3 μM) alone or with test compounds. The data shown were derived from one experiment performed in duplicate and are typical of three independent experiments giving similar results. The k_{-1} values calculated from three independent experiments are listed in the text.

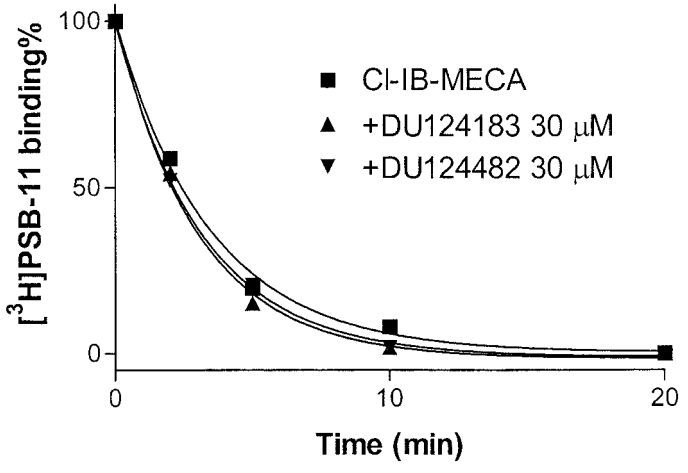
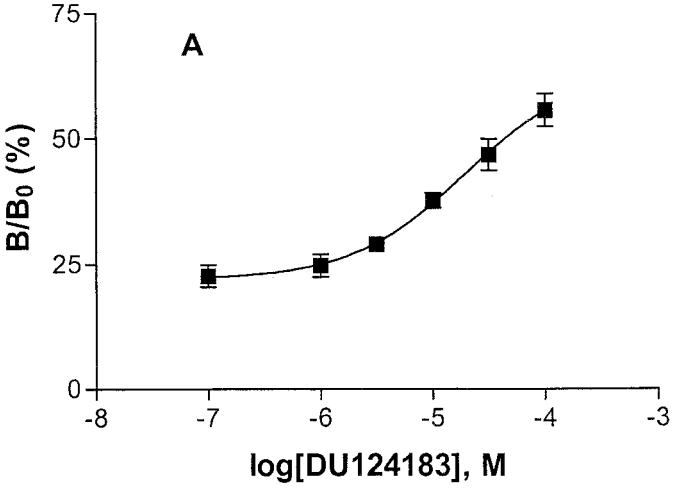


Fig. 3. Time course of the dissociation of [^{3}H]PSB-11 from the human A_3 adenosine receptor expressed in CHO cells. Membranes (80 μg) were preincubated with 8 nM [^{3}H]PSB-11 in a total assay volume of 200 μl for 60 min at 25°C. The dissociation of [^{3}H]PSB-11 from the A_3 adenosine receptor was started by addition of 3 μM Cl-IB-MECA in the absence or presence of test compounds. Nonspecific binding was measured after an incubation period of 60 min in the presence of 3 μM Cl-IB-MECA. The reaction was measured by rapid filtration at appropriate time intervals. Data are from one experiment performed in duplicate. The k_{-1} values calculated from three independent experiments are listed in the text.



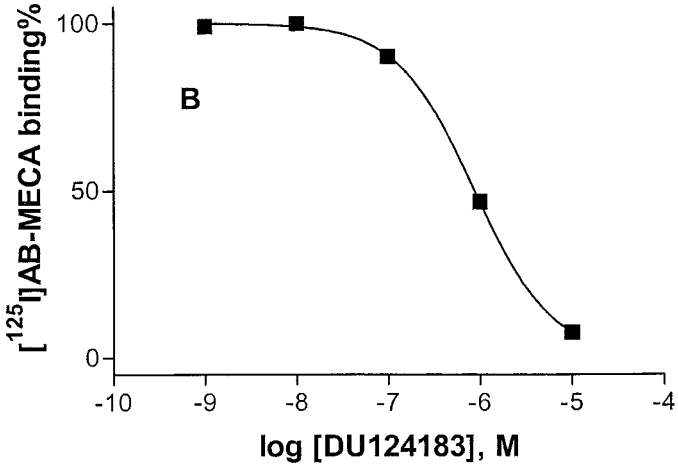


Fig. 4. Concentration-response curve for slowing the dissociation of [^{125}]I-AB-MECA by DU124183. A, [^{125}I]I-AB-MECA (1.0 nM) was preassociated with CHO cell membranes (20 μg of protein) for 60 min at 25°C without additions (total binding) or in the presence of 3 μM Cl-IB-MECA (nonspecific binding). At the end of the incubation period, 3 μM Cl-IB-MECA was added simultaneously with vehicle or various concentrations of the test compound. The incubation was terminated after an additional 45 min. Control specific binding at the end of a 45-min period of dissociation was approximately 1000 cpm, approximately 20% of the total binding. Data are mean values from three independent experiments performed in duplicate. B, dose-response curve of DU124183 competition for [^{125}I]I-AB-MECA (1 nM) binding to membranes from CHO cells expressing human A_3 receptors. The data were from one experiment performed in duplicate and are typical of three independent experiments giving similar results. The K_i values listed in Table 1 were calculated from three independent experiments.

presence of DU124183 (30 μM) and DU124482 were 0.029 ± 0.04, and 0.032 ± 0.05 min^{-1} and 0.029 ± 0.05 min^{-1} which were not significantly different ($p > 0.05$). The dissociation of the antagonist [^{3}H]ZM241385 from rat A_{2A} adenosine receptors was also not significantly affected (Fig. 6b).

Competitive Binding of 1H-Imidazo-[4,5-*c*]quinolines at A_3 Receptors. Besides their allosteric effect, the 1H-imidazo-[4,5-*c*]quinoline derivatives were also found to be competitive binding antagonists at A_3 receptors. The effect of one of the 1H-imidazo-[4,5-*c*]quinolines, DU124183, on the equilibrium binding of [^{125}I]I-AB-MECA binding was shown in Fig. 4b. The K_i values of various compounds versus [^{125}I]I-AB-MECA binding are listed in Table 1.

The effects on affinity, as orthosteric antagonist, at human A_3 receptors also depended on the substitution patterns at the 2-, 3-, and 4- positions of the imidazoquinoline derivatives. The structure activity relationships seemed to be different from those for allosteric modulation. The most potent derivatives in competitive binding experiments were the 2-phenyl-4-phenyloxy derivative DU124480 (K_i, 120 nM) and the relatively simple 4-chloro derivative DU124276 (K_i, 160 nM). Among the most potent derivatives for allosteric modulation, the 2-cyclopentyl-4-phenylamino derivative DU124183 was only of intermediate affinity in the competitive binding assay. Two other derivatives, DU124182 and DU124184, which had large enhancing effects, were more potent than DU124183 in orthosteric binding. Addition of a 3-methyl group in DU124585 reduced binding affinity of the parent phenoxy derivative, DU124182, by 120-fold. Addition of a *N*-cyclopentyl group to a 4-amino substituent had no effect on orthosteric affinity in the case of the pair of 2-phenyl derivatives, DU124482 and DU124483, although it reduced affinity by 18-fold in the case of the pair of 2-cyclopentyl derivatives, DU124184 and DU124185. Thus, for A_3 receptor competitive binding affinity, the effects of substitution at the 2- and 4-positions were also interdependent.

Functional Assay. The dissociation kinetics can provide the clearest indication of allosteric modulation; however, the potential therapeutic effect of an allosteric agent is determined by its effect on the functional activity of agonists, including the endogenous ligand. The inhibition of forskolin-

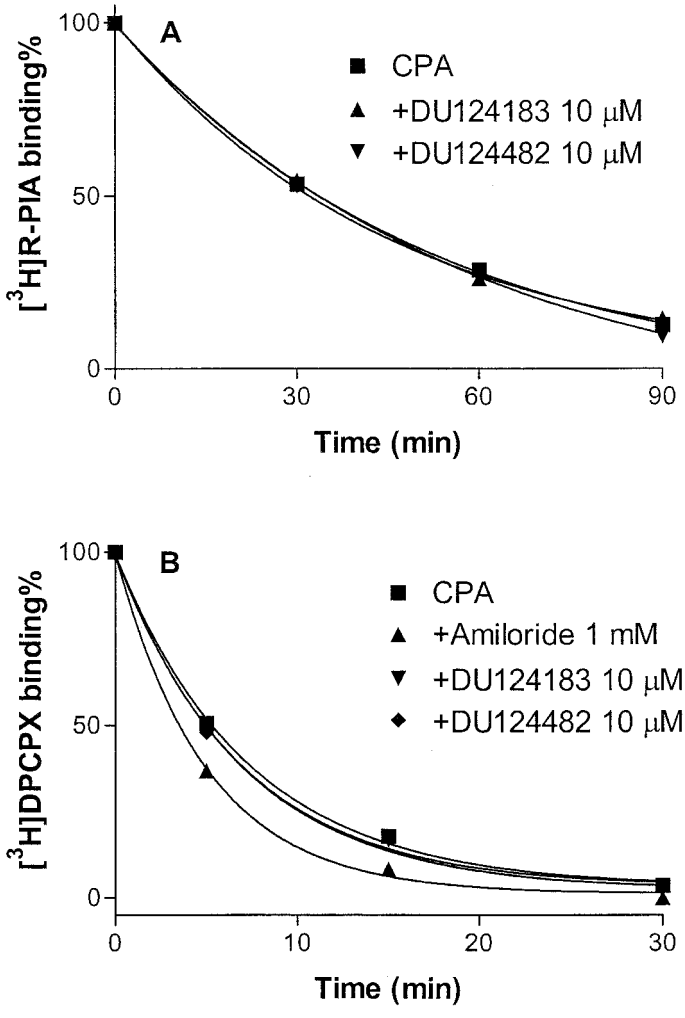


Fig. 5. Time course of the dissociation of [^{3}H](*R*)-PIA (A) and [^{3}H]DPCPX (B) from the rat brain A_1 adenosine receptors. The procedures used were described as under *Experimental Procedures*. The data shown were derived from one experiment performed in duplicate and are typical of three independent experiments giving similar results. The k_{-1} values listed in the text were calculated from three independent experiments.

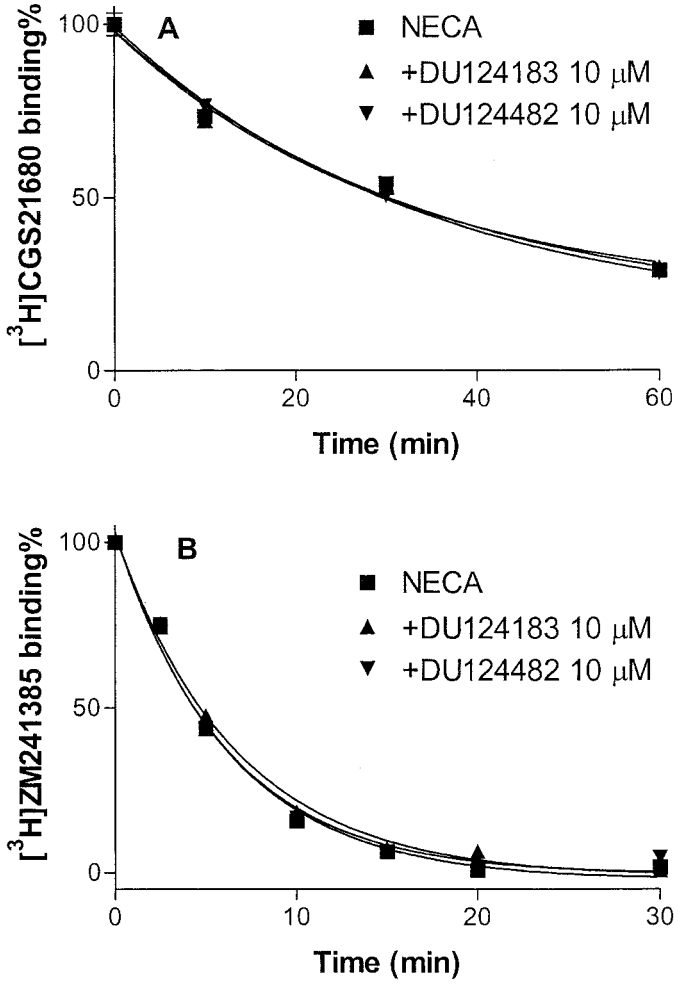


Fig. 6. Time course of dissociation of [^{3}H]CGS21680 (A) and [^{3}H]ZM241385 (B) from A_{2A} adenosine receptors. Rat striatal membranes (80 μg) were incubated with 15 nM [^{3}H]CGS21680 at 25°C for 90 min in 400 μl of 50 mM Tris-HCl, pH 7.7, containing 10 mM $MgCl_2$. NECA (10 μM) was used to define nonspecific binding. Dissociation was started by the addition of 10 μM NECA in the presence and absence of test compounds. For the dissociation of [^{3}H]ZM241385 (1 nM), the procedures used were similar to that of [^{3}H]CGS21680. The data shown were from one experiment performed in duplicate. The k_{-1} values listed in the text were calculated from three independent experiments.

stimulated cyclic AMP accumulation in CHO cells that express human A_3 receptors was employed as a functional index of A_3 receptor activation. To further evaluate the pharmacological effects of the 1H-imidazo-[4,5-*c*]quinoline derivatives, we observed the effects of these compounds on the potency and efficacy of Cl-IB-MECA, a potent and selective A_3 agonist containing ribose, and MRS1898, a newly synthesized potent and selective A_3 agonist containing a rigid (*N*)-methanocarba ring in place of the ribose moiety (Lee et al., 2001).

Both Cl-IB-MECA and MRS1898 inhibited forskolin-stimulated cyclic AMP accumulation in CHO cells expressing human A_3 adenosine receptors and showed similar maximal effects (approximately 50% of forskolin-stimulated cyclic AMP accumulation) (Fig. 7). The competitive A_3 antagonist MRS1220 blocked these inhibitory effects. In contrast to the effects of A_3 agonists, DU124183 had no direct effects on forskolin-stimulated cyclic AMP production in CHO cells expressing human A_3 receptors in the absence of an A_3 agonist (Fig. 7). The selective A_3 receptor antagonist MRS1220 (Jacobson et al., 1997) at 100 nM shifted the agonist concentration-response curves to the right but had no direct effect on cyclic AMP production or the maximum effect of agonist-induced inhibition of forskolin-stimulated cyclic AMP production. The EC_{50} values of Cl-IB-MECA were 2.1 ± 0.4 nM under control conditions and 440 ± 60 nM in the presence of 100 nM MRS1220.

Theoretically, the allosteric enhancers should induce a leftward shift in the concentration-response curve for agonist. However, this effect might be obscured by the antagonistic effects of the 1H-imidazo-[4,5-*c*]quinolines, which should produce a shift to the right. To avoid this confusing effect, the effect of DU124183 on the concentration-response curve of Cl-IB-MECA was studied in the presence of 100 nM MRS1220 (~100 times its K_i value) to overwhelm the competitive effects of DU124183. Under this condition, DU124183 caused a marked shift to the left of the Cl-IB-MECA concentration-response curve. Besides shifting the curve for Cl-IB-MECA to the left, DU124183 also potentiated the maximum inhibitory effect of Cl-IB-MECA by up to 30% (Fig. 8a). DU124482, which is closely related chemically, had no such effect. This phenomenon was also demonstrated when using MRS1898, a potent and selective full A_3 agonist (Fig. 8b). The EC_{50} values of A_3 agonists in the absence and presence of allosteric modulators are listed in Table 2.

Because of the presence of adenosine deaminase in the assay system, the further demonstration of the enhancing effect of the adenosine, which also presents endogenously, by DU124183 was not possible. However, we observed the effect of DU124183 on functional effects of CADO (2-chloroadenosine), which is the moderately potent analog that is chemically closest to the endogenous adenosine. An increase of the maximum efficacy of CADO by DU124183 was also observed in the cyclic AMP functional assay (Fig. 9). In contrast to the effect of DU124183, another allosteric modulator, VUF5455, which has been reported previously (Gao et al., 2001), did not

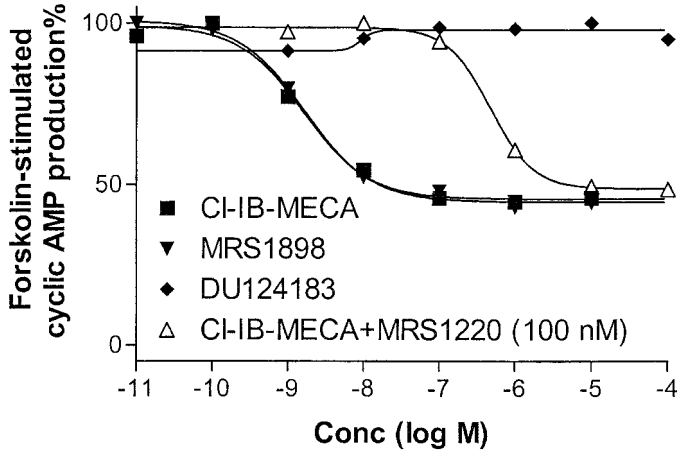


Fig. 7. Agonist-induced inhibition of forskolin-stimulated cyclic AMP production in CHO cells stably transfected with human A_3 receptors. All experiments were performed in the presence of rolipram (10 μM) and adenosine deaminase (3 units/ml). Forskolin (10 μM) was used to stimulate cyclic AMP levels. The data shown were from one experiment performed in duplicate and are typical of three independent experiments giving similar results. The level of cyclic AMP in CHO cells expressing human A_3 receptors stimulated by 10 μM forskolin was 220 ± 30 nM (n = 5). The maximal inhibition of forskolin-stimulated cyclic AMP accumulation was ~50% for agonists tested.

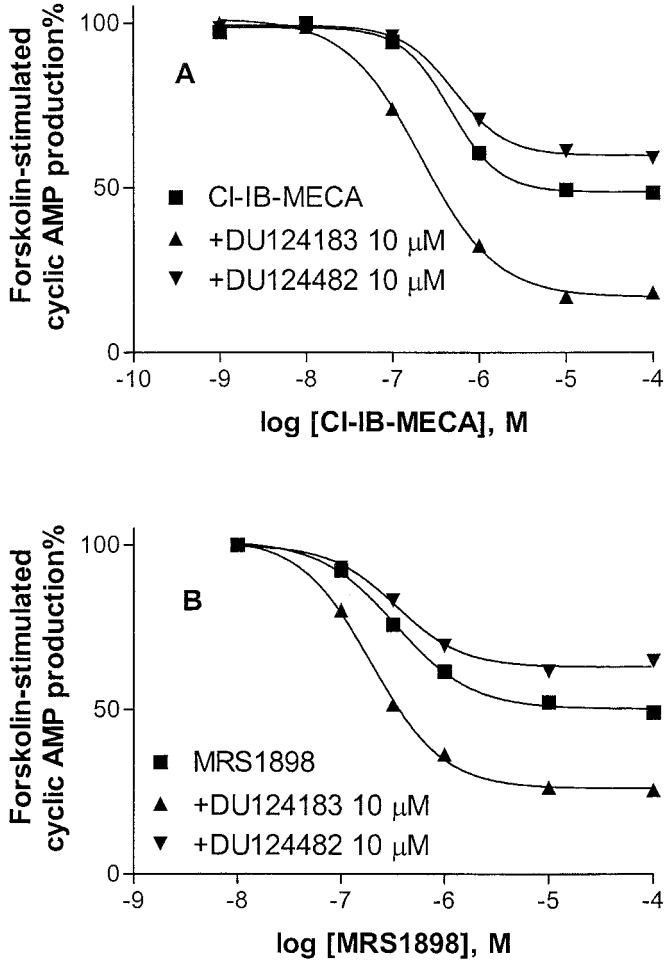


Fig. 8. Effect of DU124183 and DU124482 on the A_3 agonists Cl-IB-MECA- (A) and MRS1898 (B)-induced inhibition of forskolin-stimulated cyclic AMP production in CHO cells expressing human A_3 receptors. MRS1220 (100 nM) was used in the experiment to overwhelm the competitive effect by test compounds. Forskolin (10 μM) was used to stimulate cyclic AMP levels. All experiments were performed in the presence of 10 μM rolipram and 3 units/ml adenosine deaminase. The data are from a single experiment that was representative of three independent experiments. The EC_{50} values listed in Table 2 were calculated from three independent experiments performed in duplicate.

influence the maximum efficacy of CADO (Fig. 9), suggesting the unique mechanism of action of DU124183.

Because the 1H-imidazo-[4,5-*c*]quinolines have both antagonistic and allosteric enhancing effects, the use of an antagonist in functional assays is a useful means of discriminating enhancing from antagonistic functional effects of these compounds. However, the addition of antagonist in the assay system might obscure the important question of whether this class of compounds will functionally enhance signaling by adenosine receptor agonists through A_3 receptors in an antagonist-free environment. Hence, additional functional cyclic AMP assays in the absence of the A_3 receptor antagonist were performed. As shown in Fig. 10, one of the enhancers, DU124183, shifted the dose-response curves of A_3 receptor agonists to the right but increased the maximum effects, consistent with the mixed allosteric and competitive effects of this compound and also consistent with the enhancement of the maximum efficacy A_3 agonists in the presence of a competitive antagonist. By contrast, VUF5455 did not influence the maximum efficacy (Fig. 10).

TABLE 2

Effects of two allosteric modulators on the inhibition of cyclic AMP production by three adenosine receptor agonists in intact CHO cells expressing human A_3 receptors. MRS1220 (100 nM) was used to overwhelm the antagonistic activities of the allosteric modulators. The concentration of DU124183 and DU124482 used in the experiment was 10 μM. The accumulation level of cyclic AMP in CHO cells expressing human A_3 receptors stimulated by 10 μM forskolin was 220 ± 30 nM (n = 5). Results were expressed as mean ± S.E.M. from three independent experiments.

	EC_{50}	Cyclic AMP Level	Maximum Efficacy
	nM	*nM*	*% inhibition*
C1-IB-MECA	2.1 ± 0.4	103 ± 8	53
+ MRS1220 (control)	440 ± 60	106 ± 12	52
+ MRS1220 + DU124183	210 ± 40*	35 ± 6*	84
+ MRS1220 + DU124482	480 ± 50	130 ± 11*	41
MRS1898	1.9 ± 0.3	108 ± 9	51
+ MRS1220 (control)	350 ± 60	106 ± 10	52
+ MRS1220 + DU124183	170 ± 40*	42 ± 7*	81
+ MRS1220 + DU124482	370 ± 50	132 ± 17*	40
CADO (control)	3700 ± 780	108 ± 9	51
+ DU124183 (10 μM)	N.D.	48 ± 6*	78
+ VUF5455 (10 μM)	N.D.	110 ± 11	50

N.D., not determined.
* $P < 0.05$ compared with control.

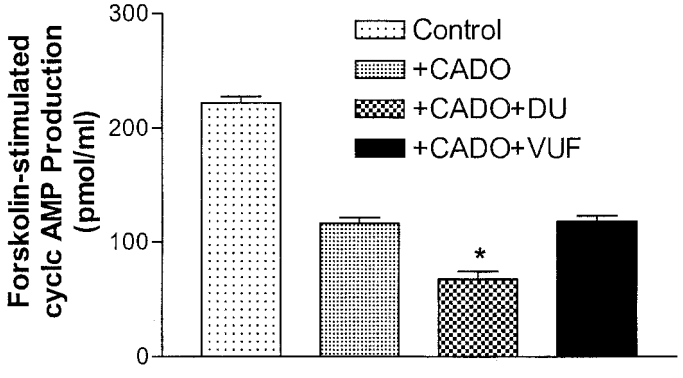


Fig. 9. Effects of DU124183 (10 μM) on the maximum effect of adenosine receptor agonist CADO (100 μM) inhibited cyclic AMP accumulation stimulated by forskolin. Forskolin-stimulated cyclic AMP level in the absence of agonist or test compound was 220 ± 30 nM. Data were from independent experiments performed in duplicate. DU, DU124183; VUF, VUF5455. *, $p < 0.05$ compared with the cyclic AMP levels of control and in the presence of 100 μM CADO.

Discussion

In the present study, we demonstrated for the first time that the imidazoquinoline derivative DU124183 selectively decreased the dissociation rate of the agonist radioligand from human A_3 adenosine receptors. Furthermore, and surprisingly, this derivative increased the maximum efficacy as well as the potency of A_3 agonists in a cyclic AMP functional assay in intact CHO cells expressing human A_3 receptors. This compound modified the intrinsic efficacy of the orthosteric ligands without having any intrinsic efficacy of its own; thus, we have identified a novel structural lead for developing allosteric enhancers of A_3 adenosine receptors.

With this set of analogs, it was possible to discern structure activity relationship patterns for the allosteric modulation of A_3 agonist effects. Additional analogs would need to be synthesized and tested to verify these tentative conclusions. Substitution at the 2- and 4-positions, however, was possible; in some cases, this resulted in enhancement of allosteric modulation. Both amino and aryl ether substituents were

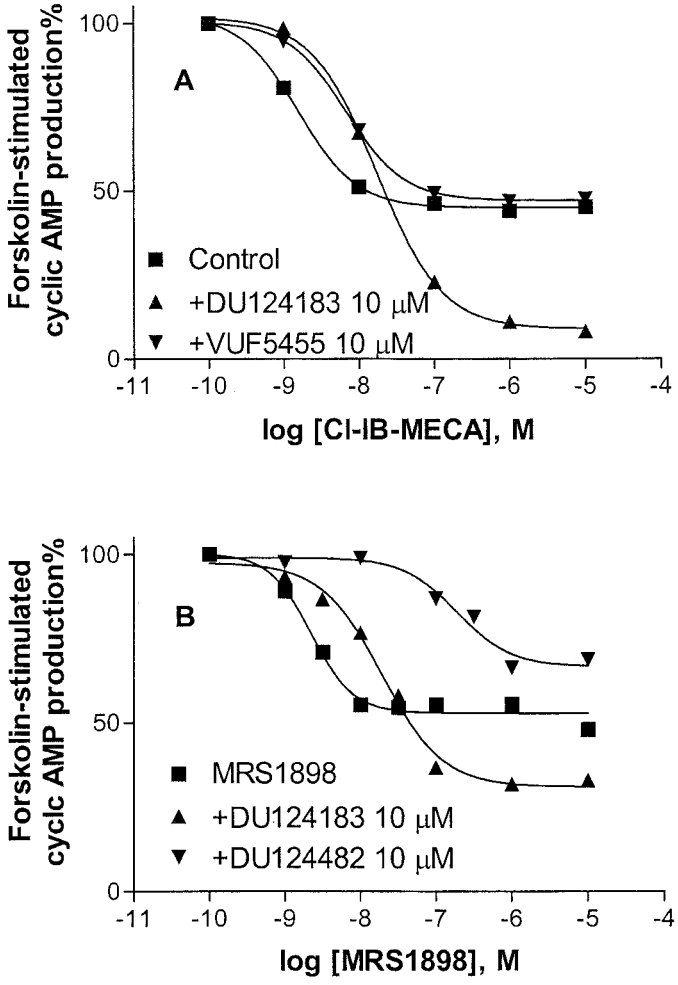


Fig. 10. Effect of allosteric enhancers (imidazoquinazolines DU124183 and DU124482 and pyridiylisoquinoline VUF5455) on the inhibition of forskolin-stimulated cyclic AMP production induced by A_3 agonists Cl-IB-MECA- (A) and MRS1898 (B) in CHO cells expressing human A_3 receptors. Forskolin (10 μM) was used to stimulate cyclic AMP levels. All experiments were performed in the presence of 10 μM rolipram and 3 units/ml adenosine deaminase. The data are from one experiment that was representative of three independent experiments performed in duplicate.

tolerated at the 4-position. A 4-phenylamino group was roughly equivalent to a 4-phenylether group in its positive effect on allosteric modulation. The effects of substitution at the 2- and 4-positions were interdependent. In the case of 4-cyclopentylamino substitution, the substituent at the 2-position may be various bulky groups. In the case of 4-phenylamino- or 4-phenyloxy-substition, the substituent at the 2-position may be cyclopentyl but not phenyl. A 3-methyl group precluded allosteric modulation by the imidazoquinoline derivative DU124182.

The most promising leads from the present set of analogs seem to be the 2-cyclopentyl-1H-imidazo[4,5-*c*]quinoline derivatives DU124182, DU124183, and DU124184. Of these analogs, DU124183 had the most favorable degree of allosteric modulation versus receptor antagonism. The compound DU124482, structurally close to DU124183, influenced the agonist dissociation rate only slightly. Furthermore, in contrast to DU124183, DU124482 decreased the maximum efficacy of agonists in the cyclic AMP functional assay without affecting agonist potency, suggesting the chemical specificity of these effects. It is interesting that the reversal of positions of the phenyl group and cyclopentyl groups resulted in an almost completely different modulatory activity.

These imidazoquinoline derivatives were first reported as antagonists of A_1 and A_{2A} receptors (van Galen et al., 1991). The imidazoquinoline analogs were tested in competitive binding assays at human A_3 receptors, and K_i values ranging from 120 nM to 101 μM were observed; as for many allosteric modulators of GPCRs, an orthosteric competitive effect was also present. One of the compounds, DU124482, was suggested to be a selective A_1 receptor antagonist (van Galen et al., 1991); the present study further confirmed this selectivity by virtue of weak affinity at A_3 receptors.

Allosteric modulation of adenosine receptors has been demonstrated in A_1, A_{2A}, and A_3 adenosine receptors (Bruns and Fergus, 1990; Gao and IJzerman, 2000; Gao et al., 2001). Positive allosteric modulators at G protein-coupled receptors have been identified in both A_1 and A_3 adenosine receptors (Bruns and Fergus, 1990; Gao et al., 2001). However, those modulators increased the agonist potency only in binding and functional assays without affecting the maximal response. For example, the allosteric enhancer PD81723 increased the potency of the A_1 receptor agonist CPA without influencing its maximum effect (Bruns and Fergus, 1990). Similarly, the isoquinoline derivative VUF5455 increased the potency of A_3 agonists but did not affect the maximum efficacy (Gao et al., 2001). These effects can be described a ternary allosteric model in which both the orthosteric and allosteric ligands bind to the receptor simultaneously and modulate reciprocally. In the case of DU124183 and DU124482, it might be more complex. Recently, Hall (2000) described an extension of the two-state model of receptor activation to account for the allosteric modulators affecting the agonist affinity as well as the intrinsic efficacy of agonists. In that model, it was suggested that the most suitable assay system may be one with very low receptor expression in which even highly efficacious agonists are unable to fully activate the signal transduction cascade (Hall, 2000). Fortuitously, in our cyclic AMP functional assay system, none of the A_3 agonists tested inhibited forskolin-stimulated cyclic AMP accumulation by more than approximately 50 to 60%. This less-than-complete inhibition made the cyclic AMP assay an ideal model for the characterization of the functional aspects of the allosteric modulators, especially the characterization of the maximum effect in the presence of allosteric modulators.

Similar to the present results demonstrated in the A_3 adenosine receptor, it has recently been reported that two chemical series of compounds, including Ro 01-6128, act as positive allosteric modulators for mGlu1 receptors (Knoflach et al., 2001), and 2,6-di-*tert*-butyl-4-(3-hydroxy-2′,2′-dimethyl-propyl)phenol (CGP7930) (Urwyler et al., 2001) as allosteric modulators for $GABA_B$ receptors. These allosteric modulators also increased both the affinity and the maximum efficacy. However, Litschig et al. (1999) reported that an allosteric modulator for the mGlu1 receptor, 7-hydroxyiminocyclopropan[*b*]chromen-1a-carboxylic acid ethyl ester, decreased the efficacy of glutamate in stimulating phosphoinositide hydrolysis without affecting its potency.

The present results show that there were distinct structural requirements for allosteric enhancement of A_3 adenosine receptor binding, and these requirements were different from those for competitive A_3 antagonistic activity. For example, DU124183 was 3-fold less potent than DU124482 but it was more potent than DU124482 in its allosteric enhancing effect. It seems that the structure-activity relationships for allosteric enhancement are separable from those for competitive antagonism, suggesting that it may be possible to discover compounds with improved enhancing activity that lack antagonist activity. Nevertheless, in the present study, in a functional assay, the efficacy-enhancing effects of DU124183 were evident at high concentrations of agonist and even in the absence of the antagonist MRS1220.

In summary, we identified a new chemical series of compounds as allosteric enhancers for A_3 adenosine receptors. The 2-cyclopentyl-4-phenylamino analog DU124183 potentiated both the potency and maximal efficacy of an agonist-induced response. Allosteric enhancers of A_3 adenosine receptors may be useful for treating ischemia and other conditions involving local energy deficits, and the unusual dual effects on adenosine receptor activation might open a new route in development of drugs for the ischemic diseases.

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