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Cytotoxic purine nucleoside analogues bind to A₁, A_{2A} and A₃ adenosine receptors

Kyle Jensen^{a,b}, L'Aurelle A. Johnson^{a,b,c}, Pamala A. Jacobson^{b,c}, Sonja Kachler^d, Mark N. Kirstein^{b,c}, Jatinder Lamba^{b,c}, and Karl-Norbert Klotz^d

^aDepartment of Experimental and Clinical Pharmacology, University of Minnesota, Minneapolis, MN, U.S.A

^bDivision of Pediatric Epidemiology and Clinical Research, University of Minnesota, Minneapolis, MN, U.S.A

^cComprehensive Cancer Center, University of Minnesota, Minneapolis, MN, U.S.A

^dInstitut für Pharmakologie und Toxikologie, Universität Würzburg, Würzburg, Germany

Abstract

Fludarabine, clofarabine and cladribine are anti-cancer agents which are analogues of the purine nucleoside adenosine. These agents have been associated with cardiac and neurological toxicities. Because these agents are analogues of adenosine, they may act through adenosine receptors to elicit their toxic effects. The objective of this study was to evaluate the ability of cytotoxic nucleoside analogues to bind and activate adenosine receptor subtypes (A₁, A_{2A}, A_{2B}, and A₃). Radioligand binding studies utilizing chinese hamster ovary cells, stably transfected with adenosine A₁, A_{2A}, or A₃ receptor subtype, were used to assess the binding affinities of these compounds, whereas adenylyl cyclase activity was used to assess the binding to A_{2B} receptors. Clofarabine and cladribine both bound to the A_{2A} receptor with a K_i of 17 and 15 μM, respectively. Clofarabine was the only adenosine analogue to bind to the A₃ receptor with a K_i of 10 μM, and none of these compounds bound to the A_{2B} receptor. Results show that clofarabine, cladribine and fludarabine bind to the A₁ receptor. In addition, clofarabine, cladribine, and fludarabine were A₁ agonists (IC₅₀ 3.1, 30 and 30 μM, respectively). Neither pyrimidine nucleoside analogues, gemcitabine nor cytarabine, associated with any of the adenosine receptor subtypes (K_i > 100μM). This is the first report of an interaction between all adenosine receptor subtypes and chemotherapeutic nucleoside analogues commonly used in the treatment of cancer. Therefore, activation of these receptors may be at least one mechanism through which fludarabine-associated toxicity occurs.

Keywords

Adenosine receptors; purine analogues; A₁; A_{2A}; A_{2B}; A₃ ligands; fludarabine; clofarabine; cladribine

Introduction

Adenosine is a ubiquitous purine nucleoside found in the extracellular space and generally exerts cytoprotective effects by accumulating in response to tissue damage and stress

Correspondence: L'Aurelle Johnson, PhD, MS, University of Minnesota, College of Pharmacy, Department of Experimental and Clinical Pharmacology, 308 Harvard ST SE, 7-115C WDH Minneapolis, MN, Phone: 00+1* 612 624 5430, Fax: 00+1*612 625 3927, Joh02745@umn.edu.

(Sitkovsky et al. 2004). Elevated adenosine levels promote tissue protection and repair by increasing available oxygen, protecting against ischemic damage, initiating anti-inflammatory responses, and stimulation of angiogenesis (Linden 2005). However, there are reports of adenosine receptor stimulation also linked to heightened tissue damage (Cunha 2005, Picano and Abbracchio 2000). These biologic processes are primarily mediated through the seven-transmembrane G-protein coupled adenosine receptors (GPCR) which include A₁, A_{2A}, A_{2B}, and A₃ receptor subtypes occurring in a tissue specific manner (Fredholm et al. 2001, Jacobson and Gao 2006, Ralevic and Burnstock 1998). Signaling events upon receptor binding modulate the production of intracellular cyclic adenosine monophosphate (cAMP) through G-protein effects on adenylyl cyclase. Agonists binding to A₁ and A₃ receptors inhibit adenylyl cyclase activity thereby decreasing cAMP while both A₂ receptor subtypes stimulate adenylyl cyclase promoting cAMP formation (Fredholm et al. 2001, Gessi et al. 2008).

Considered an inhibitory neuromodulator, extracellular adenosine has an important role in the central nervous system where it modulates cognition, sleep, locomotion, anxiety, and memory (Ribeiro et al. 2002). Due to a wide range of effects, adenosine receptors have become a focal point for targeted therapies in numerous CNS diseases including Parkinson's disease, epilepsy, and ischemic brain damage (Cunha 2005, de Mendonca et al. 2000, Fredholm et al. 2001, Picano and Abbracchio 2000, Ribeiro et al. 2002).

The anti-metabolite class of cytotoxic agents includes the adenosine based purine and pyrimidine nucleoside analogues, which are widely used in the treatment of hematological malignancies (Robak et al. 2005) and as part of preparative regimens for hematopoietic cell transplantation (Barker et al. 2003). These agents, cladribine, clofarabine, and fludarabine are deoxyadenosine nucleoside analogues (Figure 1) and require intracellular phosphorylation to their triphosphate moieties for activity. These compounds have similar mechanisms of action whereby the triphosphate moieties are incorporated into DNA and/or RNA, thus, inhibiting ribonucleotide reductase, DNA polymerases, and promoting apoptosis (Hentosh and Peffley 2010, Vasova et al. 1997, Zhenchuk et al. 2009).

One of the complications associated with fludarabine is a progressive and severe neurotoxicity (Merkel et al. 1986, Spriggs et al. 1986, Von Hoff 1990, Warrell and Berman 1986). Usually noted with high dose therapy (fludarabine >40mg/m²/day), the onset of neurological symptoms typically begins weeks after the final dose, and is often irreversible and fatal (Cheson et al. 1994). Protection from fludarabine neurotoxicity in mice has been associated with reduced uptake of fludarabine across the luminal membrane of the blood-brain-barrier (BBB) with the addition of 5'-phosphate of nitrobenzylthioinosine (NBMMP-P), an inhibitor of the equilibrative nucleoside transporter 1 (ENT1) (Adjei et al. 1992). Currently, the mechanisms precipitating these neurotoxic events are unknown. With evidence that fludarabine is able to cross the BBB (Lindemalm et al. 1999) the present study evaluated the ability of fludarabine and other cytotoxic nucleoside analogues to interact with the adenosine receptors, possibly identifying a mechanism of neurotoxicity.

Material and methods

Chemicals

Fludarabine (2-fluoroadenine-9-β-D-arabinofuranoside, F-ara-A, the unphosphorylated form), clofarabine (2-chloro-2'-arabino-fluoro-2'-deoxyadenosine), cladribine (2-chloro-2'-deoxyadenosine), cytarabine (cytosine β-D-arabinofuranoside), and gemcitabine (2,2'-difluoro-2'-deoxycytidine) were obtained from Sigma-Aldrich (St. Louis, MO). Fludarabine monophosphate (2-fluoroadenine-9-β-D-arabinofuranoside-5 phosphate) was obtained from Fischer Scientific (Pittsburgh, PA). [³H]CCPA (2-chloro-N⁶-cyclopentyladenosine), and

[³H]NECA (N-ethylcarboxamidoadenosine) were obtained from GE Healthcare, Freiburg, Germany, [³H]HEMADO (2-(1-Hexynyl)-N-methyladenosine) was from Tocris, Bristol, UK, [-³²P]ATP was from Hartmann-Analytic, Braunschweig, Germany.

Cell Culture

Chinese hamster ovary (CHO) cells were grown adherently and maintained in Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM) and geneticin (G418, 0.2 mg/ml; A_{2B}, 0.5 mg/ml) at 37°C in 5% CO₂/95% air. For binding assays the culture medium was removed, cells washed with PBS and frozen in dishes until preparation of membranes.

Membrane Preparation

Membranes from CHO cells, stably transfected with one human adenosine receptor subtype, were removed from petri dishes via scraping in the presence of ice-cold hypotonic buffer (5mM Tris/HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized on ice (Ultra-Turrax, 2 × 15 s at full speed) followed by low speed centrifugation (1,000 × g). A second, high speed centrifugation (100,000 × g) separated a crude membrane fraction from supernatant. Crude membrane was resuspended in buffers specific for each receptor subtype, frozen in liquid nitrogen, and immediately stored at -80°C. For measurement of adenylyl cyclase activity, a slightly modified protocol with only one centrifugation step was used. Fresh cells were homogenized and the homogenate centrifuged for 30 min at 54,000g. The resulting pellet was resuspended in 50 mM Tris/HCl pH 7.4 and used for the adenylyl cyclase assay immediately.

Adenosine Receptor Radioligand Binding Assay

Competition binding experiments were employed to determine K_i values of the purine analogues (fludarabine, F-ara-A, clofarabine, and cladribine) and the pyrimidine analogues (gemcitabine and cytarabine) for the adenosine A₁, A_{2A} and A₃ receptor subtypes. Briefly, competitors for radioligand binding experiments were 1 nM [³H]CCPA for A receptors, 30 nM [³H]NECA for A_{2A} receptors, and 1 nM [³H]HEMADO for A₃ receptors. Crude membranes expressing A₁, A_{2A}, or A₃ receptor subtypes were incubated with the specified radioligand and 0-100 μM fludarabine or F-ara-A, 0-30 μM clofarabine, 0-100 μM cladribine, 0-100 μM cytarabine or 0-100 μM gemcitabine in 96 well microplates with filter bottoms for 3h at 25°C. After filtration membranes were washed with cold binding buffer to remove unbound ligand. Following the addition of scintillation fluid, samples were counted using a Wallac Micro-Beta counter. With no high-affinity ligands available for A_{2B} receptor, potency and efficacy of compounds were assessed through their effect on adenylyl cyclase activity (Klotz et al. 1998). As expected, the phosphorylated form of fludarabine had very high K_i values therefore not all receptor subtypes were completed for fludarabine phosphate and all other experiments were conducted with F-ara-A.

Adenylyl Cyclase Activity

To determine the agonist or antagonist properties of fludarabine (F-ara-A), clofarabine, and cladribine, CHO membranes transfected with one receptor subtype (A₁, A_{2A}, or A_{2B}) were incubated with approximately 150,000 cpm of [-³²P]ATP and either 0-100 μM F-ara-A, 0-30 μM clofarabine, 0-100 μM cladribine for 20 min in the incubation mixture as previously described (Klotz et al. 1985) without EGTA, and NaCl (only stimulatory experiments). As agonist controls 1 μM CCPA (A₁) or 1 μM NECA (A_{2A}) were used.

Data Analysis

K_i values from competition experiments were determined utilizing the program SCTFIT. For agonists the EC_{50} -values for the stimulation of adenylyl cyclase were calculated with the Hill equation. IC_{50} -values for concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase via A_1 receptors were calculated accordingly. All mean values are from at least three experiments and given with 95% confidence intervals.

Results

Adenosine receptor radioligand binding affinity of nucleoside analogues

The binding affinities of cladribine, clofarabine, and F-ara-A to the A_1 , A_{2A} , and A_3 adenosine receptors were assessed by radioligand competition binding. All three compounds showed the highest affinity for the A_1 receptor (Table 1). The rank order of affinity at A_1 receptors was clofarabine > cladribine > fludarabine (Figure 2A). Clofarabine and cladribine bound with low affinity to the A_{2A} subtype while only clofarabine showed detectable binding to the A_3 receptor (Table 1). Figure 2B illustrates the affinity of clofarabine as determined in competition binding assays at A_1 , A_{2A} and A_3 receptors. Due to the lack of a useful radioligand the interaction with the A_{2B} subtype was only tested in adenylyl cyclase experiments (see below). Fludarabine monophosphate and the pyrimidine nucleoside analogues gemcitabine and cytarabine, showed no detectable affinity for any adenosine receptor subtype (data not shown).

Adenylyl cyclase activity

Modulation of adenylyl cyclase activity, as measured by [^{32}P]cAMP formation from [^{32}P]ATP, was determined for the inhibitory A_1 receptors and both stimulatory A_2 subtypes. All interacting compounds were determined to be full agonists at the A_1 receptor as they inhibited cAMP production in a concentration dependent manner to the same extent as the prototypical A_1 agonist CCPA (Figure 3). The IC_{50} value for clofarabine was 3.1 μM and above 10 μM for both cladribine and fludarabine (Table 2). As commonly observed for the inhibitory adenosine receptor subtypes IC_{50} values for cyclase inhibition are lower than binding K_D values by at least one order of magnitude.

Figure 4 shows that clofarabine and cladribine are partial agonists at the A_{2A} adenosine receptor with about 50% and 40% efficacy, respectively, compared to the full agonist NECA. As no measurable binding affinity from our previous experiments was detected at the A_{2A} receptor subtype, fludarabine was not tested in cyclase experiments. As expected, EC_{50} values were in the same order of magnitude as the K_D values from binding experiments (Tables 1, 2).

There was no A_{2B} -mediated activation of adenylyl cyclase found by any purine analogue. EC_{50} values for all compounds were > 100 μM (Table 2). No inhibition of NECA-stimulated cyclase activity was detected by concentrations of 100 μM , demonstrating that these compounds are not antagonists at the A_{2B} receptor (not shown).

Discussion

Fludarabine, cladribine, and clofarabine are adenosine analogues that bind to various degrees to all adenosine receptor subtypes except the A_{2B} receptor. Previous studies in murine models have demonstrated that the nucleoside analogue, cladribine, is a partial agonist of the adenosine receptor subtype A_1 (Lorenzen et al. 1998). Additionally, other murine models have demonstrated that fludarabine can alter natural killer cell activity through adenosine receptors (Priebe et al. 1990). This study demonstrates an interaction

between human adenosine receptor subtypes and adenosine analogues which are readily used in the treatment of cancer.

The overall role of adenosine in the central nervous system is very complex. It plays an important role in physiological as well as pathophysiological situations (Dunwiddie and Masino 2001). Stimulation of A₁ adenosine receptors is considered to be neuroprotective, most importantly through the presynaptic inhibition of excitatory neurotransmitters (Dunwiddie and Masino 2001, Fredholm et al. 2005). Neuroprotection is also achieved with A_{2A} receptor antagonists, probably by blockade of presynaptic receptors that enhance release of excitatory neurotransmitters (Fredholm et al. 2005). The situation is further complicated as data show that A_{2A} agonists also offer neuroprotection in some experimental models. These effects might be mediated indirectly via non-neuronal targets (Fredholm et al. 2005).

Because fludarabine, cladribine, and clofarabine exhibit close similarity to adenosine they might elicit unwanted side effects through interaction with adenosine receptors. For example, it is well documented in hematopoietic cell transplant that 2-10% of patients experience mild to severe neurological toxicities associated with fludarabine (Beitinjaneh et al. 2011, Chun et al. 1986, Ding et al. 2008). However, patients receiving either clofarabine- or cladribine-based regimens do not experience these neurological side effects. One plausible mechanism for fludarabine associated neurotoxicity is through interaction with the adenosine A₁ receptor. This receptor is primarily located in the brain and is highly expressed in neurons. Over activation of the A₁ receptor induces sleep and coma, whereas inhibition may increase the occurrence of seizures; thus the downstream effect of activation or inhibition results in neurological responses such as epilepsy, sleep, and neurological ischemia (Sebastiao and Ribeiro 2009). In the case of fludarabine, we have determined that F-ara-A binds with agonistic properties to the A₁ receptor at plasma concentrations previously determined to be clinically relevant (Long-Boyle et al. 2011b). We also determined that clofarabine and cladribine are also agonists of the A₁ receptor at concentrations previously determined to be clinically relevant (Long-Boyle et al. 2011a). The binding affinities for the A₁ receptor were clofarabine > cladribine > fludarabine. Clofarabine (10 μM) was observed to be more efficacious in decreasing adenylyl cyclase activity than equivalent concentrations of fludarabine or cladribine. Our data demonstrate that clofarabine has a higher affinity than cladribine and fludarabine at the A₁ receptor site. However, this finding is contradictory to the fact that clofarabine and cladribine have a safer neurological toxicity profile when compared to fludarabine (Faderl et al. 2005). Both, clofarabine and cladribine exhibit some affinity for A_{2A} receptors that involves partial agonistic activity. It is possible that A₁ stimulation together with a partial A_{2A} effect may afford neuroprotection which is absent in the case of fludarabine with lower A₁ affinity and no interaction with A_{2A} receptors. The combined A₁- and A_{2A}-mediated effects on cAMP levels are hard to predict as they depend on the endogenous tonic stimulation of all subtypes. In particular in a situation with higher adenosine levels a partial blockade of A_{2A} receptors by clofarabine and cladribine might potentiate the A₁-mediated decrease of cAMP and thereby provide neuroprotection that is absent with fludarabine. In addition, alternative mechanisms for fludarabine induced neurotoxicity may exist. One possibility is that these compounds may mediate their neurotoxic effects through direct activation of intracellular signaling pathways, bypassing the adenosine receptors.

Ultimately the ability for either of these agents to cause neurological effects is dependent on their ability to penetrate the blood brain barrier. Autoradiography studies in mice have shown that tritiated adenosine-like compounds (such as clofarabine, fludarabine, and cladribine) distribute into the brain; however, fludarabine uptake into the brain is ~2.5 times higher than both cladribine and clofarabine (Lindemalm et al. 1999). Furthermore animal

studies have demonstrated that fludarabine neurotoxicity can be decreased by reducing its uptake across the blood brain barrier (BBB) by NBMPPR-P, an ENT1 uptake transporter inhibitor (Adjei et al. 1992). Despite cladribine and clofarabine's higher binding affinities to the A₁ receptor relative to fludarabine, poor penetration across the BBB would likely limit their ability to accumulate brain concentrations approximate to their K_i values. Hence, poor brain penetration may be one explanation for the lack of neurotoxicity associated with these two compounds.

There is also an abundance of A₁ receptors in the heart (Fredholm et al. 2001). However rare, fludarabine induced grade IV/V cardiotoxicity has been observed in four separate studies in patients who were undergoing nonmyeloablative hematopoietic cell transplant and received fludarabine (Martino et al. 2001, Ritchie et al. 2001, Van Besien et al. 2003). Alternatively, interaction of clofarabine and cladribine with the A_{2A} receptor may be protective towards the neurological effects associated with the activation of A₁ receptor thus, making clofarabine and cladribine more suitable when attempting to avoid the neurological complications which are associated with fludarabine.

In conclusion these results suggest that clofarabine, cladribine and fludarabine bind as agonists to adenosine receptors with various affinities. Even though these compounds bind A₁, one must account for their limited CNS penetration, which could ultimately restrict their ability to cause neurotoxicity, especially cladribine and clofarabine. Further studies are needed to fully determine the molecular pathways involved in the cytotoxic nature of these agents in order to develop compounds with reduced toxicities.

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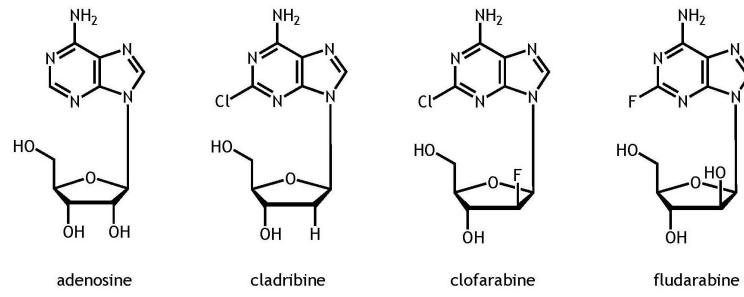


Figure 1. Chemical structures of adenosine and the analogues cladribine, clofarabine, and fludarabine.

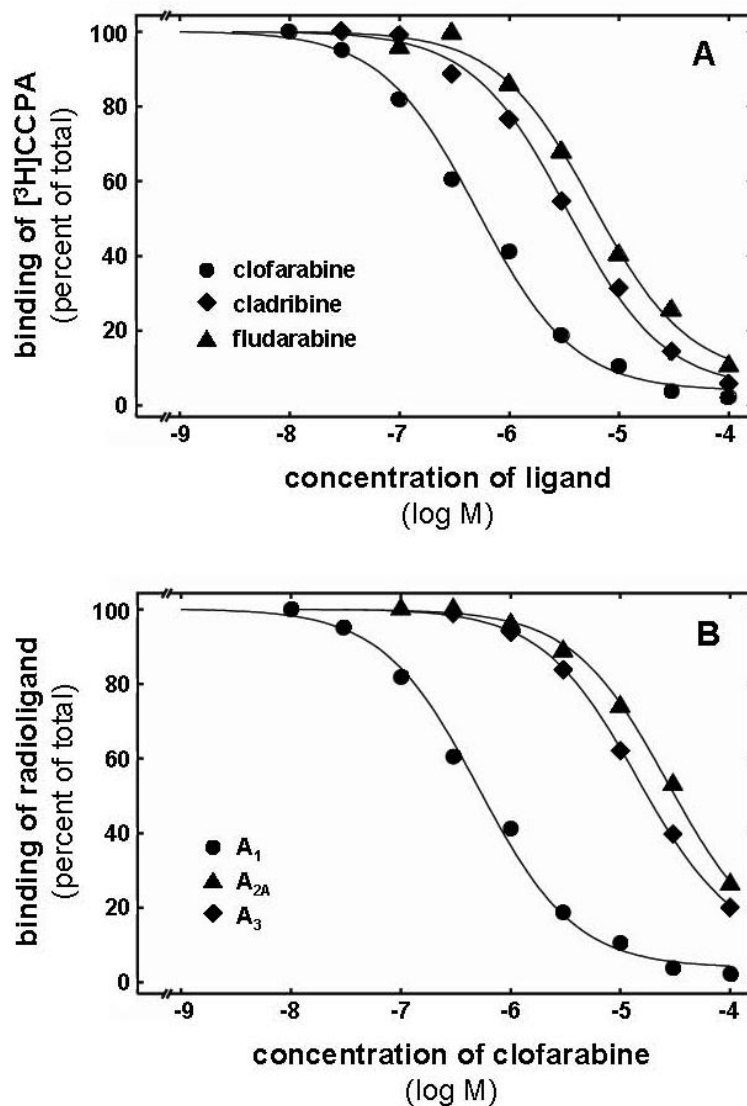


Figure 2. Radioligand competition experiments with clofarabine, cladribine and fludarabine. The curves in A represent competition for $[^3\text{H}]\text{CCPA}$ binding to A_1 adenosine receptors with clofarabine showing the highest affinity (K_i 248 nM), followed by cladribine (K_i 1,643 nM) and fludarabine (K_i 2,238 nM). In B competition binding of clofarabine to A_1 , $\text{A}_{2\text{A}}$, and A_3 receptors using $[^3\text{H}]\text{CCPA}$, $[^3\text{H}]\text{NECA}$, and $[^3\text{H}]\text{HEMADO}$ as radioligands, respectively (corresponding K_i values are: 248 nM, 17,500 nM, and 8,280 nM). The shown curves are from single experiments, for data in detail see Table 1.

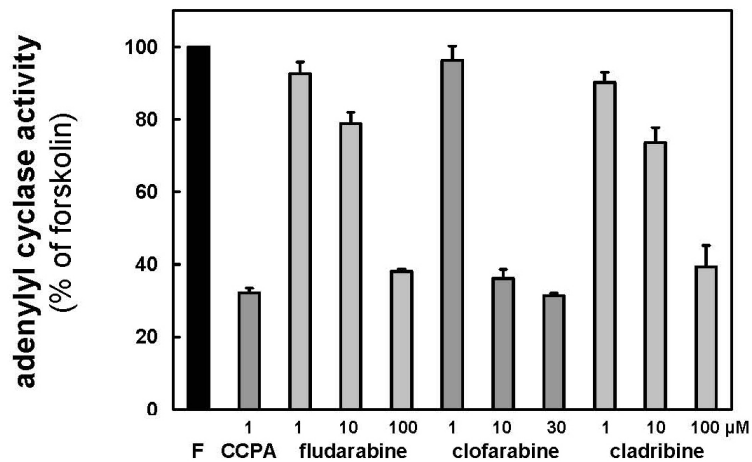


Figure 3. Inhibition of adenylyl cyclase activity via stimulation of A₁ receptors. Adenylyl cyclase was stimulated with forskolin (F) and concentration-dependent inhibition by fludarabine, clofarabine and cladribine is shown. The maximal inhibition is comparable to the full A₁ receptor agonist CCPA. Data represent percent of forskolin-stimulated cyclase activity over basal (n = 3, error bars show SEM).

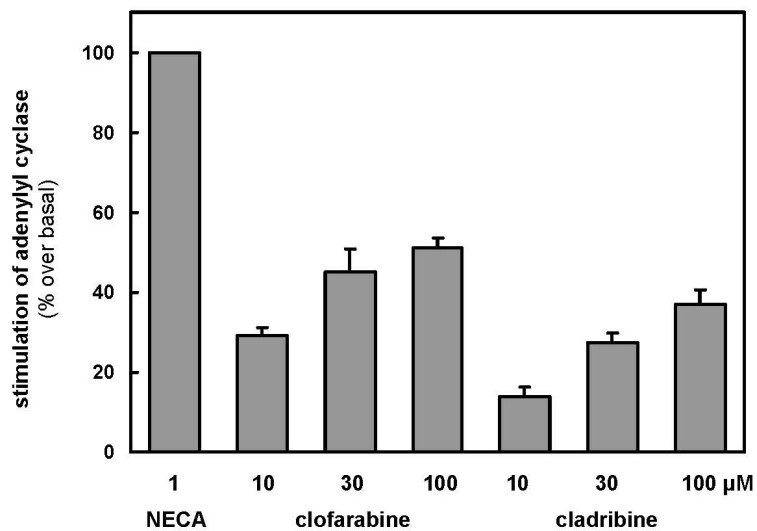


Figure 4. A_{2A} receptor-mediated stimulation of adenylyl cyclase with clofarabine and cladribine. Both compounds are partial agonists with about 50% and 40% efficacy, respectively, compared to the full agonist NECA. Error bars represent SEM, $n = 3$

Table 1

K_i values for clofarabine, cladribine and fludarabine at A_1 , A_{2A} , and A_3 receptor subtypes. Values are K_i from radioligand binding studies given in nM with 95% confidence intervals in parentheses (n=3).

	A_1	A_{2A}	A_3
clofarabine	274 (224-335)	17,100 (10,900-26,900)	10,600 (7,190-15,800)
cladribine	1,570 (1,450-1,710)	15,100 (11,300-20,200)	> 100,000
fludarabine	3,000 (2,330-3,870)	> 100,000	> 100,000

Table 2

Effect of clofarabine, cladribine and fludarabine on adenylyl cyclase activity. Values are IC₅₀ (EC₅₀ for A_{2B}) given in nM with 95% confidence intervals in parentheses (n=3).

	A ₁	A _{2A}	A _{2B}
clofarabine	3,130 (1,890-5,190)	9,800 (8,120-11,800)	> 100,000
cladribine	> 10,000	16,100 (10,500-24,700)	> 100,000
fludarabine	> 10,000	n.d.	> 100,000

n.d., not determined